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Detection and control of brucellosis in reindeer vaccinated with *Brucella suis* biovar 3

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DETECTION AND CONTROL OF BRUCELLOSIS IN REINDEER VACCINATED WITH *BRUCELLA SUIS* BIOVAR 3

A

THESIS

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DETECTION AND CONTROL OF BRUCELLOSIS

IN *BRUCELLA SUIS* BIOVAR 3-VACCINATED REINDEER

by

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ABSTRACT

The objective of this research was to provide a vaccine for the control of brucellosis in reindeer, that allows serologic discrimination between vaccinated and infected animals. Three vaccines were tested: (1) *Brucella suis* 1, (2) *B. suis* 3, and (3) A rough mutant of the infective strain, *B. suis* 4. All were heat-killed and prepared in Freund's incomplete adjuvant. Each vaccine was administered to four animals. All vaccines stimulated the production of high levels of antibody in *Rangifer* that were maintained for the 483-day experiment. Significant delayed-type hypersensitivity reactions were seen in all vaccinated *Rangifer*. Both *B. suis* 1 and *B. suis* 3 vaccines allowed serologic discrimination between vaccinated and infected *Rangifer*. This was accomplished by means of an indirect ELISA (enzyme-linked immunosorbent assay). This test used whole cell *B. melitensis* and *B. abortus* as A and M-dominant antigens. Distinction could be made between vaccinated and infected reindeer based on a percentage difference in spectrophotometric absorbance values obtained with these antigens. The *B. suis* 3 vaccine provided the best discrimination. Eighty-nine percent of 117 reindeer were correctly classified as either *B. suis* 3-vaccinated or *B. suis* 4-infected. Discrimination between vaccinated and infected reindeer was sufficient to allow assessment of the prevelance of brucellosis in vaccinated herds. In addition, the ELISA was more sensitive than standard agglutination tests in identifying reindeer with exposure to *B. suis*. The *B. suis* 3 vaccine was further evaluated in a challenge of 7 vaccinated reindeer. The vaccinated group consisted of 5 pregnant adults and 2 8-month-old female calves. These reindeer were challenged with 3.16×10^7 colony forming units of *B. suis* 4 at 63 days post-vaccination. Five pregnant adults and 1 female calf served as

experimental controls. *B. suis* 4 was isolated from 3 of 7 vaccinated reindeer (43%) at the time of necropsy. *B. suis* 4 was isolated from the aborted fetus of 1 of the infected vaccinates. Another infected vaccinate bore a healthy calf for which *B. suis* 4 could not be isolated. All control reindeer were infected and all 5 adults aborted. *B. suis* 4 was isolated from all 5 fetuses. The *B. suis* 3 vaccine provided significant protection against infection and abortion in reindeer challenged with *B. suis* 4.

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INTRODUCTION

Brucellosis is an infectious disease of mammals including humans, caused by bacteria of the genus *Brucella*. Most species of domestic animals are susceptible to infection by one or more species of *Brucella*. Distribution of the disease is world-wide (Thimm 1982).

BRUCELLOSIS IN REINDEER

Brucellosis due to *Brucella suis* biovar 4 is enzootic in populations of reindeer and caribou *(Rangifer tarandus)* throughout polar regions of the world (Meyer 1966). These animals are considered the primary host of *B. suis* 4. The bacteria causes reproductive losses through abortion and the birth of weak calves (Davidov 1961, Neiland et al. 1968). Additionally, the disease causes orchitis, epididymitis and sterility in males (Golosov and Zabrodin 1959), and arthritis and bursitis with accompanying lameness in both sexes (Davidov 1961, Nikolaevskii 1961, Orloff 1963).

TRANSMISSION

The primary mode of transmission of *B. suis* 4 in reindeer and caribou is believed to be via ingestion of aborted membranes and uterine discharges (Davidov 1961). Animals may become incidentally infected when feeding on a contaminated site. Bacteria remain viable on the ground under arctic conditions for extended periods of time (Vaskevich 1973).

DIAGNOSIS

Antemortem diagnosis of brucellosis in *Rangifer* is based upon appearance of clinical signs and presence of *Brucella*-specific antibodies in the bloodstream. Abortion is the most common sign of the disease, but is difficult to observe in herded reindeer. Externally visible lesions are rare. Only 1-5% of reindeer in an infected herd may show obvious lesions (Cherchenko 1961). Serologic tests currently available for the diagnosis of brucellosis in reindeer accurately identify recently infected animals, but may fail to identify chronically infected ones (Golosov and Zabrodin 1959, Dieterich 1981). Isolation of the bacterium from reindeer tissues is the only way to confirm a diagnosis. This step usually requires the slaughter and necrospy of reindeer.

ANTIBODY PREVALENCE OF BRUCELLOSIS IN ALASKAN REINDEER HERDS

Brucellosis is enzootic in most Alaskan reindeer herds. Antibody prevalence in reindeer herds ranges from <1% to >20% (Dieterich 1981). Similar prevalences have been reported by Russian researchers. Davidov (1974) reported that 11.1% of 1,756 reindeer from Yakutia had serologic evidence of exposure to *B. suis*. Under such circumstances losses in herd productivity through abortion could be expected. Russian researchers have reported that abortion and barrenness range from 11% to 30% in *Brucella*-infected herds (Golosov and Zabrodin 1959).

CONTROL OF REINDEER BRUCELLOSIS

Control of brucellosis in Alaskan reindeer herds is sought for three reasons. First, herd productivity would be expected to improve where brucellosis levels are kept to a minimum. Under a suitable control program, reindeer herders would be able to maximize antler and meat harvests. Second, the risk of human infection to those working

within the reindeer industry would fall to negligible levels. Third, export markets for live reindeer would become available with the ability to assure brucellosis-free animals.

Brucellosis in domestic animals has been eradicated in many western countries through government-regulated test-and-slaughter programs in conjunction with the use of efficacious vaccines, where available (Alausa et al. 1986). Unfortunately, eradication of brucellosis in most herds of Alaskan reindeer is not feasible because reindeer are herded across exceptionally large areas of unfenced tundra.

Brucellosis in Alaskan herds of reindeer has been controlled with the use of a killed *B. suis* 4 vaccine. This vaccine provides protection against infection in laboratory trials (Dieterich et al. unpubl.). Results from field applications of this vaccine are also encouraging. Antibody prevalence has decreased from approximately 30% to < 5% in nonvaccinated "sentinel" animals in one vaccinated herd (Dieterich, pers. comm.).

The main disadvantage to this vaccine is that naturally-infected reindeer cannot be distinguished from vaccinated, noninfected animals. Serologic tests currently available do not discriminate between the antibody response of vaccinated reindeer and infected reindeer. This has caused difficulties in: (1) Evaluating the prevalence of brucellosis in vaccinated herds, (2) Evaluating the efficacy of the vaccine as used under field conditions, and (3) Designating vaccinated reindeer as being free of actual infection. Currently, only nonvaccinated reindeer are selected for export from *Brucella*-enzootic areas because vaccinated, noninfected reindeer cannot be serologically distinguished from infected animals (Stahmann 1991). This is contrary to optimal control of the disease because reindeer originating from vaccinated herds would be less likely to harbor the organism than animals from nonvaccinated herds.

RESEARCH OBJECTIVES

The objective of this research was to develop a vaccine that: (1) Protects reindeer from infection with *B. suis* 4, and (2) Allows serologic discrimination between vaccinated and naturally infected animals. The initial phase of this research involved a trial of three vaccines. All three were expected to elicit an antibody response, which could be distinguished from that of an infected animal. The first two vaccines were based on biovars of *Brucella* infectious for swine, namely *B. suis* biovar 1 and *B. suis* biovar 3. *B. suis* 4 has been classified in the same species as these biovars because of similarities in biochemistry, metabolism and phage susceptibility (Meyer 1964). *B. suis* 1 and *B. suis* 3 were chosen for the vaccine trial because they lack a form of O polysaccharide antigen, the M antigen, which is present in *B. suis* 4 (Alton et al. 1975, Wilson and Miles 1975). I hypothesized that reindeer vaccinated with these strains could be distinguished from those infected with *B. suis* 4 based on a differential response to this antigen. The third strain used in the trial was a rough *B. suis* 4 mutant, obtained from a laboratory at the National Animal Disease Center for the purposes of this study. I hypothesized that a vaccine based on this rough *B. suis* 4 mutant would produce an antibody response of low magnitude and short duration because this mutant lacks O polysaccharide. By contrast, animals infected with *B. suis* 4 would be expected to show an antibody response of high magnitude and short duration.

RESEARCH DESIGN

These *Brucella* vaccines were initially tested in a small group of reindeer and caribou to compare induced humoral and cellular immunity. Humoral immunity was assessed for 18 months following vaccination by regular serologic testing. Cell-mediated responses were measured by a delayed-type hypersensitivity test with a *B. suis* 4 protein allergen given intra-dermally. This research is outlined in Chapter 1.

Simultaneously, an ELISA (enzyme-linked immunosorbent assay) procedure was developed to discriminate between infected reindeer and those vaccinated with either *B. suis* 1 or *B. suis* 3. This research is presented in Chapter 2. I used serum samples derived from the vaccinated reindeer and caribou in the 3-way vaccine trial as test sera.

Six months after the initiation of the 3-way vaccine trial, I chose the most promising of the three vaccines for further testing in a challenge experiment. Criteria used for the selection of this vaccine were: (1) Humoral and cell-mediated immune responses of vaccinated *Rangifer*, and (2) Ability to discriminate between vaccinated and infected animals. The *B. suis* 3 vaccine was chosen for the challenge experiment. I hypothesized that the *B. suis* 3 vaccine would produce sufficient immunity in reindeer to protect them against challenge with virulent *B. suis* 4. In this experiment, I challenged vaccinated and control reindeer with *B. suis* 4, and later compared them with regards to infection status and the occurrence of abortions. This research is presented in Chapter 3.

I also tested the *B. suis* 3 vaccine that was chosen for the challenge experiment in a free-ranging reindeer herd with an existing prevalence of serum antibody for brucellosis of < 1%. This biovar contains the A antigen but not the M antigen, whereas *B. suis* 4 contains both antigens (Alton et al. 1975, Wilson and Miles 1932, 1975). These antigens are chemically distinct O polysaccharide chains that form part of the smooth lipopolysaccharide complex (s-LPS) of the outer membrane of smooth *Brucella* species (Bundle et al. 1987, Cherwonogrodzky et al. 1987). Thus, *B. suis* 3-vaccinated reindeer should be discriminated from *B. suis* 4-infected reindeer based on the presence or absence of antibodies directed against the M polysaccharide antigen. Antibodies directed against this antigen might be detected by means of a specifically designed ELISA. In this manner, vaccinated reindeer could be differentiated from naturally infected animals. I

hypothesized that the *B. suis* vaccine would engender sufficient immunity in reindeer to protect against challenge with virulent *B. suis* 4.

The purpose of this field test was to provide a sufficient sample size of *Brucella*-free vaccinated reindeer with which to establish the sensitivity and specificity of the discriminatory ELISA. The ELISA results from field-vaccinated reindeer are presented in Chapter 2, along with the ELISA results from 11 reindeer vaccinated with *B. suis* 3 in the vaccine trials described previously.

CHAPTER 1.

IMMUNOGENICITY OF *BRUCELLA SUIS* BIOVAR 1, *BRUCELLA SUIS* BIOVAR 3, AND ROUGH *BRUCELLA SUIS* BIOVAR 4 VACCINES IN *RANGIFER.*

ABSTRACT

I tested 3 vaccines for prevention of brucellosis in *Rangifer*: (1) *B. suis* 1, (2) *B. suis* 3, and (3) A rough mutant of *B. suis* 4, lacking smooth lipopolysaccharide. All were heat-killed and prepared in Freunds' incomplete adjuvant. I administered each vaccine to 4 animals. The humoral response elicited by the vaccines was assessed by regular serologic testing for 483 days. Cell-mediated responses were assessed by delayed-type hypersensitivity tests performed at 112 and 161 days post-vaccination. All vaccines stimulated the production of high levels of antibody in *Rangifer* that were maintained throughout the course of the experiment. Significant delayed-type hypersensitivity reactions were seen in all vaccinated *Rangifer*. I chose *B. suis* 3 vaccine for further testing and evaluation because of better discrimination.

INTRODUCTION

This chapter describes a trial of three vaccines for brucellosis in reindeer. All three vaccines were expected to produce an antibody response, which could be distinguished from that of an infected animal. Two vaccines were based on biovars of *Brucella* infectious for swine, *B. suis* biovar 1, and *B. suis* biovar 3. *B. suis* 1 and *B. suis* 3 were chosen for the vaccine trial because they contain the A antigen but not the M antigen, whereas *B. suis* 4 contains both antigens. Thus, I expected that reindeer

vaccinated with these strains could be distinguished from *B. suis* 4-infected reindeer based on a differential response to the M antigen. The third *Brucella* vaccine used in these trials was based on a rough *B. suis* 4 mutant, lacking major antigenic determinants normally present in *B. suis* 4 smooth-lipopolysaccharide (s-LPS). Here, I expected that vaccinated reindeer would show a weak and temporary antibody response as measured by standard serologic tests and enzyme-linked immunosorbent assay (ELISA), and could be distinguished from infected animals on this basis. The three vaccines were evaluated on the basis of ability to induce humoral and cell-mediated responses to *Brucella* in reindeer, and also on the degree of discrimination between vaccinated and infected reindeer.

The objective of this research was to provide criteria on which to select one of the three vaccines for further testing and evaluation. Criteria used for selection were based on outcomes from tests of the following null hypotheses: (1) *B. suis* 3 and *B. suis* 1 vaccines produce an antibody response in *Rangifer* that is similar in duration and magnitude, (2) Rough *B. suis* 4 vaccine produces an antibody response in *Rangifer* which is weak and transient, and (3) *B. suis* 3, *B. suis* 1, and rough *B. suis* 4 vaccines produce cell-mediated responses in *Rangifer* that are equivalent.

METHODS

Three *B. suis* strains were selected for evaluation as vaccines. *B. suis* 1330 is a typical biovar 1, whereas *B. suis* 636 is a typical biovar 3 isolated from swine and shown to be pathogenic for this species (Deyoe 1967). *B. suis* RAS is a laboratory-induced rough mutant of a *B. suis* 4 originally isolated from a Seward Peninsula reindeer (IAB 2579). The National Animal Disease Center, located in Ames, Iowa, provided species strains and confirmed strain designations by standard typing procedures (Alton et al. 1975). The rough mutant was typical of rough *B. suis* organisms in colony morphology, dye-exclusion characteristics and nonpathogenicity for guinea-pigs, but uncharacteristic

in behavior in suspension and in serotype. This mutant exhibited agglutination when diluted 1:200 with A monospecific serum, and exhibited incomplete agglutination when diluted 1:200 with M monospecific serum, and 1:400 with anti-rough *B. suis* serum.

The procedure used to prepare the 3 vaccines was identical to that used for the *B. suis* 4 vaccine, which currently is in use in Alaskan reindeer herds (Chapter 3). Experimental animals included groups of 3 yearling male reindeer, 3 yearling female reindeer, 3 yearling female caribou and 3 adult female caribou. These animals were housed at the Large Animal Research Station, University of Alaska Fairbanks. Vaccine treatments were allocated randomly within each group, controlling for differences in immune response relating to age, sex, and subspecies of *Rangifer*. Animals were vaccinated sub-cutaneously in the left mid-cervical area with 2 ml of vaccine containing approximately 10 mg cells per cubic weight. The experimental protocol used in this study was approved by an independent animal welfare committee at the University of Alaska Fairbanks.

The hypothesis that the *B. suis* 1 and *B. suis* 3 vaccines produce a humoral response in *Rangifer*, which was equal in duration and magnitude, was tested by sequential sampling and serologic testing of *Rangifer* treated with these vaccines. I sampled animals every week until antibody levels had peaked. After this time, I collected samples every 2 weeks. Sampling of *Rangifer* beyond 175 days post-vaccination was less intensive; animals were sampled every 30-60 days until day 483.

Serologic tests employed were standard plate (SP), buffered *Brucella* antigen (BBA), rivanol (Riv) and A antigen-based enzyme-linked immunosorbent assay (ELISA). Standard procedures of the US Department of Agriculture (U.S. Department of Agriculture not dated, b, c) were used for the SP, BBA, and Riv tests. The ELISA procedure was an indirect assay developed by Douglas et al. (1984) and modified for use

in reindeer (Chapter 2). An animal was considered positive if: (1) Agglutination occurred at a dilution of 1:25 on the SP, (2) Agglutination occurred on the BBA, (3) Incomplete agglutination occurred at a dilution of 1:25 on the Rivanol, and an optical density of 0.30 units was read on the ELISA.

Antibody curves based on geometric means were generated from the SP, Riv, and ELISA results of *B. suis* 1 and *B. suis* 3-vaccinated *Rangifer* and compared by fitting multiple regression equations (Zar 1984). Regression equations used to model the decline in SP and Riv titers after vaccination are modifications of a half-normal distribution: $Y = ae^{bx}$, where Y = log of antibody titers, e = base of the natural logarithm, and a, b = regression coefficients. Weighted fifth-order polynomial regressions were used to model the antibody response of vaccinated reindeer as measured by ELISA; a spline curve-fitting procedure was used to generate the figures. Partial *F*-Tests were used as the criterion for including an additional term in the regression equation (Zar 1984). I compared BBA test results from vaccine treatment groups using the Friedman two-way test (Zar 1984).

The second hypothesis, that the rough *B. suis* 4 vaccine would produce a weak and transient antibody response, was similarly tested by sequential serologic testing of *Rangifer* treated with this vaccine. Regressions (previously described) were fitted to geometric means from SP, Riv, and ELISA tests. These regressions were compared with regressions from *Rangifer* treated with *B. suis* 1 and *B. suis* 3 vaccines.

The third hypothesis, that the three vaccines would produce equivalent cell-mediated responses to *B. suis* 4 in vaccinated *Rangifer*, was tested by assessing the allergic reactions of vaccinated *Rangifer* to a *Brucella*-protein allergen injected intradermally. The protein allergen was prepared at the National Animal Disease Center, Ames, Iowa, from cultures of a rough mutant of *B. suis* 4 that was laboratory-induced.

The procedure employed results in a product with no detectable lipopolysaccharide (Bhongbhibhat et al. 1970, Jones et al. 1973).

I undertook delayed-type hypersensitivity trials with this protein allergen at 112 and 161 days post-vaccination. In the first trial, lyophilyzed allergen was reconstituted at a concentration of 500 g/ml. A 0.1 ml aliquot of this solution was injected intradermally in the right-mid cervical skin of vaccinated *Rangifer*. Skin-fold thickness was measured with calipers prior to injection. For this procedure, skin was grasped between thumb and forefinger and calipers were placed over the doubled skin. I measured test sites again at 24, 48 and 72 h after injection. In addition, I examined skin thicknesses at 6 h post-injection to verify that no immediate-type hypersensitivity reactions had been caused by injection of the allergen. Change in skin thickness was used to evaluate the strength of the resultant delayed-type hypersensitivity reactions. The Freidman 2-way test was used to examine differences in skin thickness between vaccine treatments.

I used a similar procedure for allergic testing in the second trial undertaken at 161 days post-vaccination. *Rangifer* vaccinated with *B. suis* 1 and *B. suis* 3 were tested, along with 2 animals with no previous exposure to *B. suis*. In this trial, skin thickness was examined at 6 and 48 h. Biopsies were taken at 48 h from skin at test sites, and from normal skin distant to test sites. Lidocaine was injected sub-cutaneously to anesthetize biopsy sites in vaccinated *Rangifer*. This local anesthetic was placed approximately 10 cm away from test sites in an L-shaped block to avoid causing edema in the test area. An alternative local anesthetic, a skin-freeze spray[1], was used in control reindeer. The biopsy site was closed with the use of a skin stapler. Biopsy tissues were fixed in 10% buffered formalin, sectioned, stained with hematoxylin and eosin, and examined microscopically. Delayed-type hypersensitivity reactions were assessed on the following

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1 Schuco, Div. A.C.I., Carle Place, NY 11514.

criteria: (1) Presence of edema, and (2) Depth of perivascular cuffing by mononuclear cells in the venular plexuses, venules, and small veins of the papillary dermis and the reticular dermis. The degree of cuffing was judged to be normal, mild, moderate or severe and ranked on a Likert scale. The Mann-Whitney *U*-test was used to examine differences in perivascular cuffing between vaccine treatment groups. Fisher's exact test was used to compare the incidence in edema in the two vaccine groups (Zar 1984).

RESULTS

A significant difference occurred among regression lines modeling the decaying serologic response of *B. suis* 1 and *B. suis* 3-vaccinated reindeer as measured by SP (F = 9.93; 3,26 df; $P < 0.001$), Riv ($F = 11.41$; 3,26 df; $P < 0.001$), and ELISA ($F = 4.34$; 6,38 df; $P < 0.005$) tests (Figures 1.1, 1.2, and 1.3). Both vaccines elicited production of similarly high levels of antibody. The *B. suis* 1 vaccine, however, produced a slightly longer duration antibody response as measured by SP and Riv tests. Conversely, the titer was greater with the *B. suis* 3 vaccine as measured in the ELISA. There was no significant difference in the serologic response of reindeer to these vaccines as measured by the BBA test ($X^2 = 0$, $P = 1.0$), which gives a categorical result (Table 1.1). The *B. suis* 1 and *B. suis* 3 vaccines did not produce antibody responses that were equivalent in duration and magnitude.

The rough *B. suis* 4 vaccine elicited production of high and long-lasting antibody levels. Serologic test results (titers) were generally lower than titers generated by the other vaccines. Regressions modeling the antibody response to rough *B. suis* 4 vaccine over time differed significantly from those produced by reindeer vaccinated with *B. suis* 1 (SP; $F = 13.42$; 3,26 df; $P < 0.001$; Riv; $F = 12.04$; 3,26 df; $P < 0.001$) and *B. suis* 3 (SP; $F = 13.74$; 3,26 df; $P < 0.001$; Riv; $F = 12.20$; 3,26 df; $P < 0.001$; ELISA; $F = 7.38$; 6,38 df; $P < 0.001$) (Figures 1.1, 1.2, and 1.3). Polynomial regressions

TABLE 1.1.
Buffered *Brucella* antigen test results for *Rangifer* vaccinated with *B. suis* 1, *B. suis* 3 and rough *B. suis* 4. Numbers of positive and negative test results in series of samples taken from each animal are presented.

	B. SUIS 1		*B. SUIS* 3		*B. SUIS* 4	
ANIMAL	POS[1]	NEG[2]	POS	NEG	POS	NEG
Yearling male reindeer	20	1	21	0	21	0
Adult female caribou	23	0	22	1	14	0
Yearling female caribou	23	0	23	0	15	8
Yearling female reindeer	22	0	22	0	22	0
TOTALS	88	1	88	1	72	8

[1] Positive test result is visible agglutination.
[2] Negative test result is lack of visible agglutination.

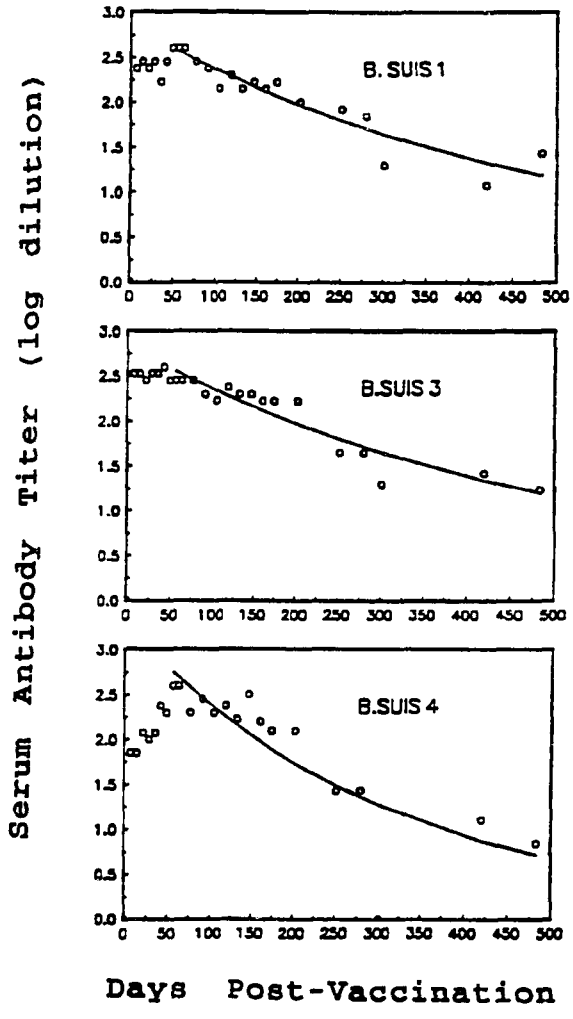

FIGURE 1.1.
Standard plate test results for three groups of 4 *Rangifer* vaccinated with *B. suis* 1, *B. suis* 3, or rough *B. suis* 4 vaccines.

$\hat{Y}$ (*B. suis* 1) = 2.840 x $e^{-0.001817X}$ $r^2 = 0.88$

$\hat{Y}$ (*B. suis* 3) = 2.814 x $e^{-0.001765X}$ $r^2 = 0.88$

$\hat{Y}$ (*B. suis* 4) = 3.281 x $e^{-0.003150X}$ $r^2 = 0.72$

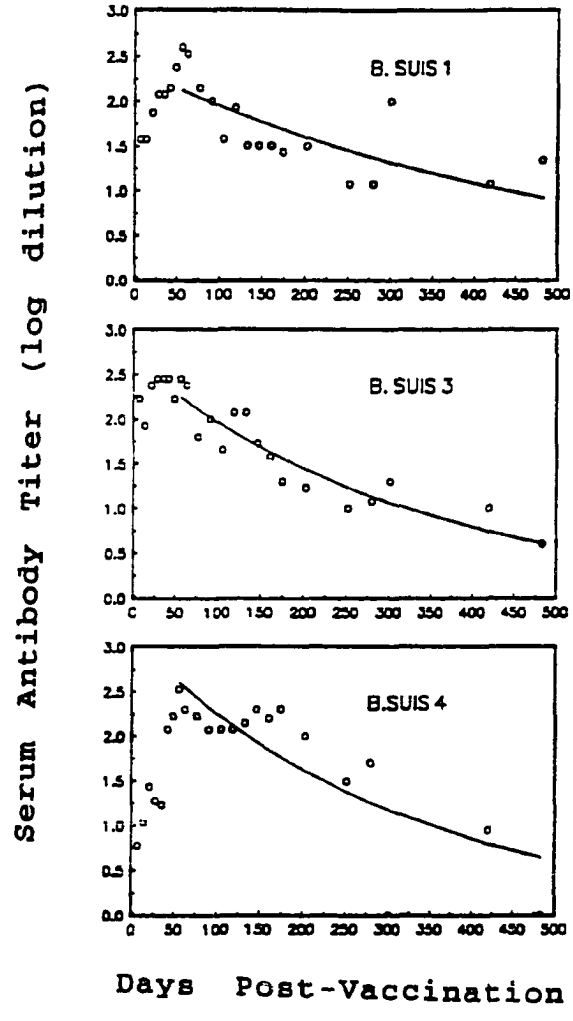

FIGURE 1.2.
Rivanol test results for three groups of 4 *Rangifer* vaccinated with *B. suis* 1, *B. suis* 3, or rough *B. suis* 4 vaccines.

$\hat{Y}$ (*B. suis* 1) = 2.369 x $e^{-0.001960X}$ $r^2 = 0.50$

$\hat{Y}$ (*B. suis* 3) = 2.662 x $e^{-0.000420X}$ $r^2 = 0.84$

$\hat{Y}$ (*B. suis* 4) = 3.129 x $e^{-0.003281X}$ $r^2 = 0.69$

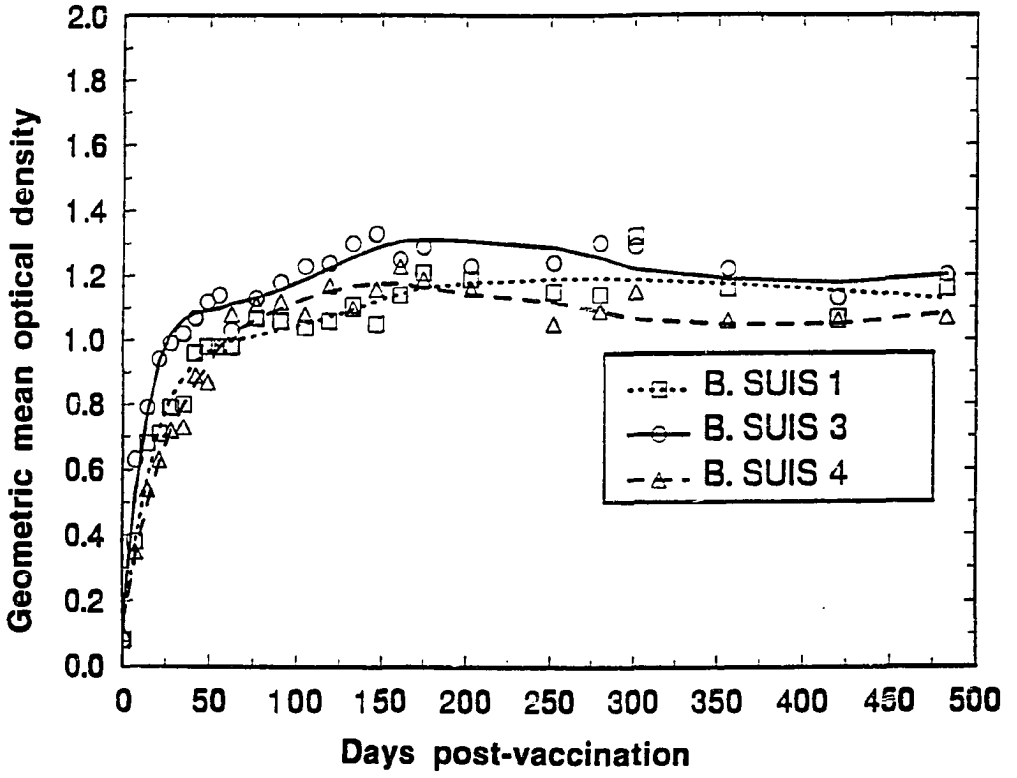

FIGURE 1.3.
Spectrophotometric absorbance values for three groups of 4 *Rangifer* spp. vaccinated with *B. suis* 1, *B. suis* 3 or rough *B. suis* 4 as measured by ELISA.

$\hat{Y}$ (*B. suis* 1) = 0.229 + $(0.251 \times 10^{-1})X - (0.266 \times 10^{-3})X^2 + (0.128 \times 10^{-5})X^3 - (0.277 \times 10^{-8})X^4 + (0.218 \times 10^{-11})X^5$.

$r^2 = 0.94$

$\hat{Y}$ (*B. suis* 3) = 0.365 + $(0.254 \times 10^{-1})X - (0.264 \times 10^{-3})X^2 + (0.123 \times 10^{-5})X^3 - (0.260 \times 10^{-8})X^4 + (0.201 \times 10^{-11})X^5$.

$r^2 = 0.88$

$\hat{Y}$ (*B. suis* 4) = 0.183 + $(0.233 \times 10^{-1})X - (0.203 \times 10^{-3})X^2 + (0.811 \times 10^{-6})X^3 - (0.152 \times 10^{-8})X^4 + (0.108 \times 10^{-11})X^5$.

$r^2 = 0.97$

modeling ELISA results from *B. suis* 1 and rough *B. suis* 4-vaccinated reindeer were not significantly different ($F = 2.13$; 6,38 df; $P > 0.2$). BBA test results from *B. suis* 4-vaccinates did not differ significantly from those of *B. suis* 1 and *B. suis* 3-vaccinated *Rangifer* ($X^2 = 0, P = 1.0$), (Table 1.1).

All three vaccines induced good cell-mediated immunity in *Rangifer* as evidenced by marked delayed-type hypersensitivity reactions to *Brucella* protein allergen. Changes in skin thickness ($X^2_{(112\ days)} = 1.14, P > 0.56$; $Z_{(161\ days)} = -0.15, P > 0.88$) and cellular changes resulting from allergen injection ($Z_{(edema)} = 0, P = 1$; $Z_{(cuffing)} = -0.61, \underline{P} > 0.54$) did not differ significantly between vaccine treatments. In the first allergic test, performed 112 days post-vaccination, the greatest changes in skin thickness were seen in *B. suis* 1-vaccinated reindeer (Table 1.2, Figure 1.4). Changes in skin thicknesses measured 48 and 72 h post-injection ranged from 0.9 to 10.5 mm in individual *Rangifer*. Measurements of skin thickness recorded at 24 h differed significantly from those taken later ($X^2 = 12.2, P < 0.002$).

In the second allergic test, changes in skin thickness at 48 h ranged from 0.5 mm to 4.0 mm (Table 1.3, Figure 1.5). All skin sections from *Rangifer* showed edema, and distinct perivascular accumulations of lymphocytes and macrophages in superficial and deep dermal layers. These results confirm the presence of delayed-type hypersensitivity reactions (Table 1.4).

Vaccinated animals maintained a differential response to A and M antigens as measured in ELISA throughout 483 days of testing (Figure 1.6). In *B. suis* 3 vaccinates, this difference remained > 20%. This contrasts to results from rough *B. suis* 4-vaccinates (Figure 1.6) where percentage differences fell to levels approximating 0 at 100 days post-vaccination. Regression equations modeling declines in percentage differences were

TABLE 1.2.
Change in skin thickness at three time periods following injection of *Brucella* protein allergen in *Rangifer* 112 days post-vaccination with *B. suis* 1, *B. suis* 3 and rough *B. suis* 4 vaccines.

VACCINE	ANIMAL	CHANGE IN SKIN THICKNESS (mm) 24 h	48 h	72 h
B. SUIS 1	AFC[1]	1.5	3.5	3.0
	YFC[2]	2.0	3.5	3.5
	YFR[3]	7.0	9.5	10.5
	YMR[4]	2.0	3.5	4.0
	MEAN	3.1	5.0	5.3
	SD[5]	2.6	3.0	3.5
B. SUIS 3	AFC	1.5	2.0	1.5
	YFC	3.0	6.0	4.5
	YFR	3.5	5.0	6.0
	YMR	2.5	2.5	2.5
	MEAN	2.6	3.9	3.6
	SD	0.9	1.9	2.0
B. SUIS 4	AFC	4.5	6.5	5.5
	YFC	3.0	2.5	3.5
	YFR	2.5	5.0	5.0
	YMR	4.5	5.0	4.5
	MEAN	3.6	4.8	4.6
	SD	1.0	1.7	0.9

[1] Adult female caribou
[2] Yearling female caribou
[3] Yearling female reindeer
[4] Yearling male reindeer
[5] Standard deviation

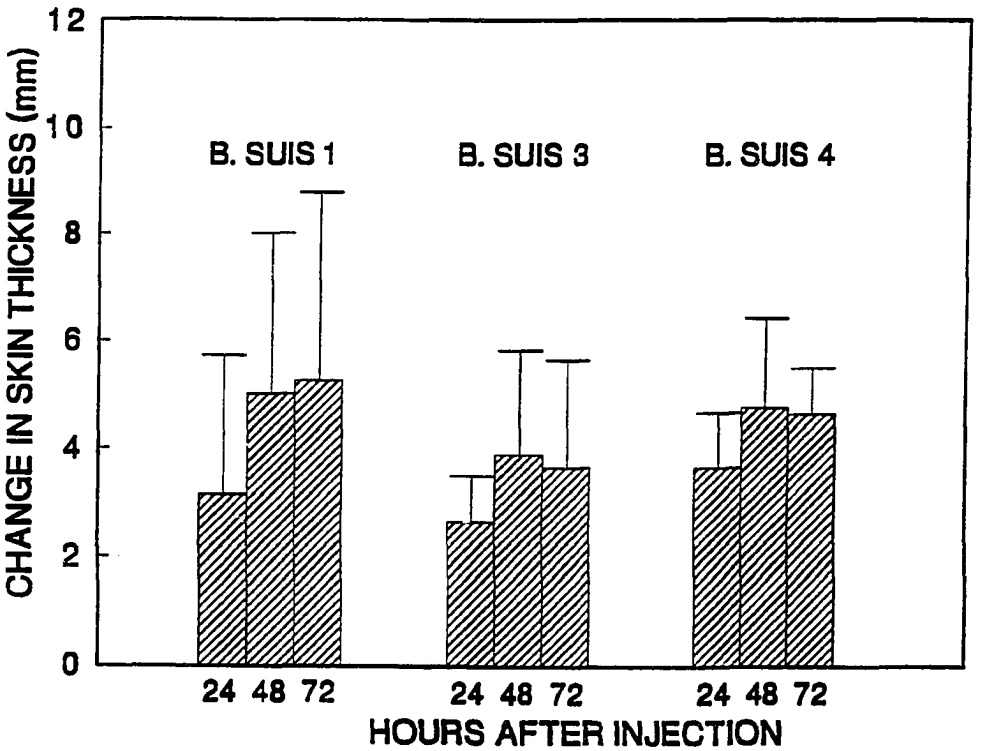

FIGURE 1.4.
Change in skin thickness (mm) in three groups of 4 *Rangifer* injected with *B. suis* 4 protein allergen 112 days after vaccination with *B. suis* 1, *B. suis* 3, and rough *B. suis* 4. Skin thickness was measured at 24, 48 and 72 hours after intradermal injection. Means ±1 standard error are presented.

TABLE 1.3.
Change in skin thickness 48 h following injection of *Brucella* protein allergen in *Rangifer* 161 days post-vaccination with *B. suis* 1 and *B. suis* 3 vaccines.

VACCINE	ANIMAL	CHANGE IN SKIN THICKNESS (mm)
B.SUIS 1	AFC1	1.0
	YFC2	2.5
	YFR3	2.5
	YMR4	3.5
	MEAN	2.4
	SD5	1.0
B. SUIS 3	AFC	0.5
	YFC	4.0
	YFR	2.5
	YMR	3.0
	MEAN	2.5
	SD	1.5

[1] Adult female caribou
[2] Yearling female caribou
[3] Yearling female reindeer
[4] Yearling male reindeer
[5] Standard deviation

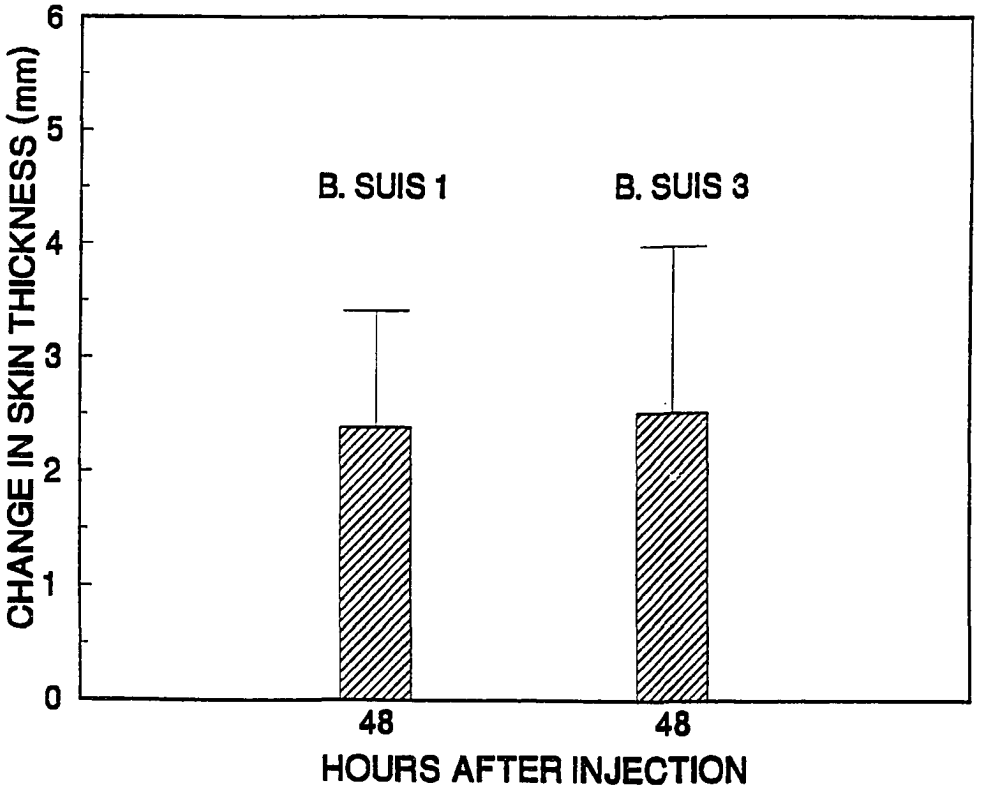

FIGURE 1.5.
Change in skin thickness (mm) in two groups of 4 *Rangifer* injected with *B. suis* 4 protein allergen 161 days after vaccination with *B. suis* 1 and *B. suis* 3. Skin thickness was measured at 48 hours after intradermal injection. Means +1 standard error are presented.

TABLE 1.4.
Cellular changes indicative of delayed-type hypersensitivity in skin biopsies taken from *Rangifer* at 48 h after injection of *Brucella* protein allergen. Biopsies were taken from normal skin in addition to skin at the test site and evaluated for the presence or absence of edema and depth of perivascular cuffing by inflammatory cells. Biopsy results from *B. suis* 1 and *B. suis* 3-vaccinated *Rangifer* tested 161 days post-vaccination are presented, along with results from control animals.

VACCINE	ANIMAL	SKIN TYPE	EDEMA	CUFFING
B. SUIS 1	AFC[1]	NORMAL	-	NORMAL
		TEST	SLIGHT	MILD
	YFC[2]	NORMAL	-	MILD
		TEST	+	SEVERE
	YFR[3]	NORMAL	-	NORMAL
		TEST	+	MODERATE
	YMR[4]	NORMAL	-	NORMAL
		TEST	+	SEVERE
B. SUIS 3	AFC	NORMAL	-	NORMAL
		TEST	+	MILD
	YFC	NORMAL	SLIGHT	MILD
		TEST	+	MODERATE
	YFR	NORMAL	-	NORMAL
		TEST	+	SEVERE
	YMR	NORMAL	-	NORMAL
		TEST	+	MILD
CONTROL	YFR	NORMAL	-	NORMAL
		TEST	SLIGHT	MILD
	YFR	NORMAL	-	NORMAL
		TEST	-	NORMAL

[1] Adult female caribou
[2] Yearling female caribou
[3] Yearling female reindeer
[4] Yearling male reindeer

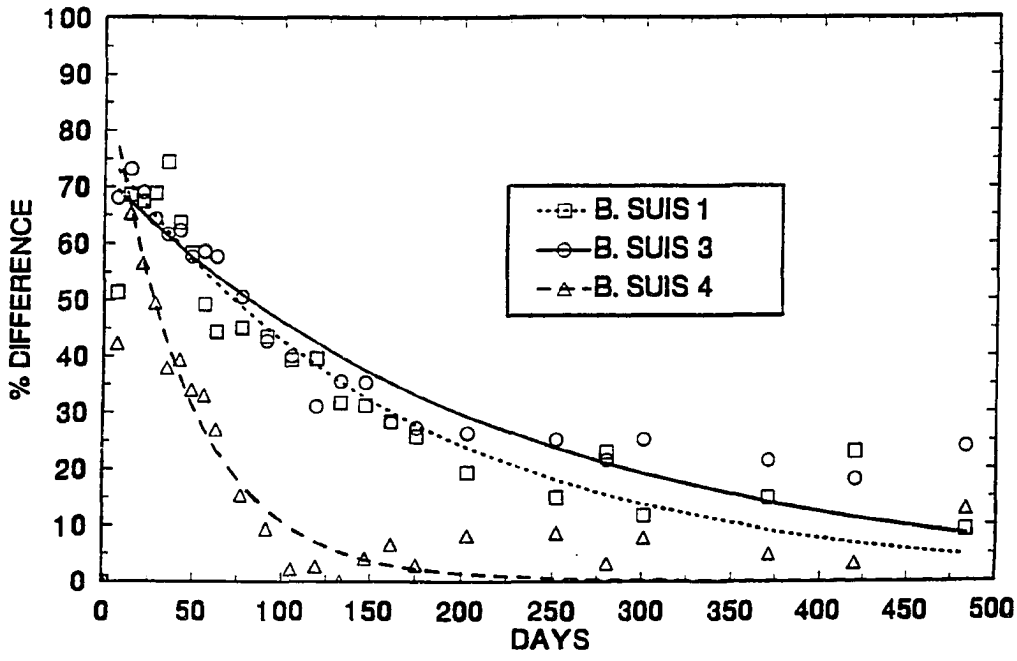

FIGURE 1.6. Percentage differences in ELISA spectrophotometric absorbance values obtained on A and M-dominant antigens [(A - M)/A x 100)] for three groups of 4 *Rangifer* vaccinated with *B. suis* 1 (a), *B. suis* 3 (b), and rough *B. suis* 4 (c) vaccines. *Rangifer* were sampled sequentially for 483 days after vaccination. Means ±1 standard error, and non-linear regression are presented.

$\hat{Y}$ (*B. suis* 1) = $75.866 \times e^{-0.005740X}$ $r^2 = 0.92$

$\hat{Y}$ (*B. suis* 3) = $71.531 \times e^{-0.004416X}$ $r^2 = 0.89$

$\hat{Y}$ (*B. suis* 4) = $89.488 \times e^{-0.021415X}$ $r^2 = 0.93$

significant among vaccine treatment groups: (a) *B. suis* 1 with rough *B. suis* 4 (F = 138.38; 3,40 df; $P < 0.001$); (b) *B. suis* 1 with *B. suis* 3 (F = 17.16; 3,40 df; $P < 0.001$) (Fig. 1.6); and (c) *B. suis* 3 with rough *B. suis* 4 (F = 167.57; 3,40 df; $P < 0.001$) (Fig. 1.6). The *B. suis* 3 vaccine provided a greater differential response to A and M antigens than did the *B. suis* 1 vaccine. Contrary to expectations, the rough *B. suis* 4 vaccine produced an antibody response of long duration and high magnitude.

DISCUSSION

All vaccinated *Rangifer* produced antibody as evidenced by high serologic titers in SP, Riv, BBA and ELISA tests. This result was desirable in *B. suis* 1 and *B. suis* 3 vaccinates, but not in *Rangifer* vaccinated with rough *B. suis* 4. There was some variation in the humoral responses of *Rangifer* treated with the three vaccines. Titers as measured by Riv rose most quickly in *B. suis* 3-vaccinates and persisted longest in *B. suis* 1-vaccinates. Titers measured using SP rose most quickly in *B. suis* 1 and *B. suis* 3-vaccinates; and titers as determined by ELISA were slightly greater in *B. suis* 3-vaccinates. Consequently, I reject the first hypothesis that the *B. suis* 1 and *B. suis* 3-vaccines would produce an antibody response that was similar in magnitude and duration.

Variation in serologic test results between treatments may be an effect of experimental design. Nonetheless, this variation also may be attributed to a differential response in the production of different antibody types between individual *Rangifer*. Only four *Rangifer* were used per treatment. Therefore, natural variability of immune response between members of the group could alter geometric means. Research has shown that reindeer, which are exposed to *Brucella* under identical conditions, produce variable humoral responses (Dieterich, pers. comm.). Variation in titers between tests can be ascribed to the presence of different classes of immunoglobulin in *Rangifer* sera. In

cattle, the SP test detects principally IgM and IgG_2, the buffered *Brucella* antigen test detects primarily IgG_1 and IgG_2, the Riv test detects primarily IgG_1 (Tizard 1987), and the ELISA is specific for reindeer IgG (Chapter 2).

Results from agglutination tests were more variable than results from ELISA. Agglutination tests present a crude system for the quantification of antibody; the observer visually inspects dilutions of serum in an antigen mixture for the presence of antibody-antigen complexes that have agglutinated (U.S. Department of Agriculture not dated, b, c). Agglutination is secondary to antigen-antibody complex formation, and assays employing this method of antibody quantization are termed a secondary-binding assays. The ELISA is a primary-binding assay that measures antigen-antibody interaction directly. Thus the higher variability of results in agglutination tests as compared to those from ELISA was not unexpected.

Rangifer vaccinated with rough *B. suis* 4 produced high levels of antibody as measured by all tests. Consequently, I reject the second hypothesis. Rough strains of *Brucella* lack polysaccharide normally present in s-LPS (Moreno et al. 1979). The polysaccharide component of LPS is important in eliciting antibody formation in exposed animals (Diaz et al. 1968). Indeed, antibodies detected in standard agglutination tests are mostly specific for O polysaccharide (Diaz et al. 1968, Schurig et al. 1981). The rough *B. suis* 4 strain used in this experiment is likely to have contained significant amounts of O polysaccharide because it elicited the production of high levels of antibody in *Rangifer* as measured in agglutination tests. This is not unusual for rough strains of *Brucella.* Degrees of "roughness" (variable coating of s-LPS) occur in dissociated cultures (Huddleson 1943), and specific rough strains possess small amounts of low molecular-weight polysaccharide (Meikle et al. 1989). In addition, this rough mutant was atypical of rough *B. suis* organisms because agglutination occurred with serum monospecific for

A and M polysaccharide. This observation lends additional support to the conclusion that the rough *B. suis* 4 contained appreciable amounts of polysaccharide.

Rough strains of *Brucella*, which have been used to control brucellosis in domestic animals, do not elicit the production of long-lasting levels of measurable antibody (Alton et al. 1975). For example, domestic cattle vaccinated with *B. abortus* strain 45/20 produce low, transient levels of antibody. Killed *B. abortus* strain 45/20 has been tested in reindeer for the prevention of brucellosis (Dieterich et al. 1981). This vaccine did not induce high antibody levels in reindeer until these animals were later challenged with virulent *B. suis* 4 (Chapter 2), producing an anamnestic or secondary antibody response. The rough mutant of *B. suis* 4 used in this experiment was inappropriate for use in a vaccine. The production of a stable rough mutant completely lacking O polysaccharide, however, could provide excellent protection from brucellosis without the persistence of serologic titers (Schurig et al. 1991).

All three vaccines induced good cell-mediated immunity in *Rangifer* as evidenced by marked delayed-type hypersensitivity reactions to *B. suis* protein allergen. The third hypothesis is therefore supported. The use of allergic tests for brucellosis diagnosis in *Rangifer* has not been reported previously. Similar techniques have been employed successfully in brucellosis diagnosis in cattle, sheep, goats and pigs (Fensterbank 1985, Nicoletti and Winter 1990, Nicoletti 1983/1984). The protein allergen appears to have been free of LPS. No rise in serologic titers was seen following administration of the allergen, and no Arthrus-type (immediate hypersensitivity) reactions were noted. This test may be useful in identifying seronegative reindeer calves that are carriers for brucellosis. The allergic test has been reported to diagnose brucellosis in calves born to infected cattle (Bercovich et al. 1990).

The antibody response to A and M antigens differed most significantly in *Rangifer* vaccinated with *B. suis* 3. For this reason, and because of greater biochemical similarities with *B. suis* 4 (Alton et al. 1975), the *B. suis* 3 vaccine was chosen for further evaluation and testing.

CHAPTER 2.

ENZYME-LINKED IMMUNOSORBENT ASSAY TO DISCRIMINATE BETWEEN *BRUCELLA SUIS* BIOVAR 4-INFECTED AND *BRUCELLA SUIS* BIOVAR 3-VACCINATED REINDEER.

ABSTRACT

Brucella suis biovar 4 is the causative agent of brucellosis in herds of Alaskan reindeer. Control of the disease has been based on the use of a killed *B. suis* 4 vaccine. Infected reindeer in vaccinated herds cannot be distinguished from vaccinated, noninfected animals by means of serologic tests currently available. A *Brucella suis* biovar 3 vaccine was tested that would allow serologic discrimination between vaccinated and infected reindeer based on a differential response to A and M polysaccharide antigens. An indirect ELISA (enzyme-linked immunosorbent assay) was developed using whole cell *B. melitensis* and *B. abortus* as A and M-dominant antigens. Epitopes shared by both A and M antigens were blocked with a monoclonal antibody, C-1. The ELISA is more sensitive than standard agglutination tests in identifying reindeer with exposure to *B. suis*. Distinction could be made between vaccinated and infected reindeer based on a percentage difference in spectrophotometric absorbance values obtained on A and M-dominant antigens. Percent difference in A and M-specific antibody response diminished with time in vaccinated reindeer. Nevertheless, the indirect ELISA classified 89% of 117 reindeer correctly as either vaccinated or infected. Discrimination between *B. suis* 3-vaccinated and *B. suis* 4-infected reindeer is sufficient to allow assessment of brucellosis prevalence in vaccinated herds.

INTRODUCTION

Brucellosis due to *Brucella suis* biovar 4 is enzootic in many herds of reindeer and caribou throughout the world (Meyer 1966). The disease is one of high morbidity and low mortality. The primary effects of the disease are reproductive loss from abortion in females and sterility in males (Golosov and Zabrodin 1959, Davidov 1961). In addition, infected reindeer frequently develop bursitis or arthritis with associated lameness (Davidov 1961, Nikolaevskii 1961, Dieterich 1981).

Most reindeer herds in Alaska are kept on unfenced ranges of up to 0.5 million hectares of mountainous tundra. Under such conditions, eradication of brucellosis with a test-and-slaughter program is not feasible. Instead, research efforts have been directed at producing a safe and efficacious vaccine that would control the disease in infected herds. A killed *B. suis* 4 vaccine has been tested in reindeer and provides an excellent level of protection (Dieterich et al. unpubl.). This vaccine is presently in use in most Alaskan herds of reindeer.

The main disadvantage to the use of this vaccine is that infected reindeer in vaccinated herds cannot be distinguished from vaccinated, noninfected animals. Visible brucellosis lesions only occur in 1-5% of reindeer in infected herds (Cherchenko 1961, Zabrodin et al. 1980). Serologic tests currently available do not discriminate between the antibody response of vaccinated and infected reindeer.

A killed *B. suis* biovar 3 vaccine was tested in reindeer. This vaccine might allow serologic discrimination between vaccinated and infected reindeer based on a differential response to the immunodominant A and M antigens of *B. suis*. These antigens are chemically distinct O polysaccharide chains that form part of the smooth lipopolysaccharide complex (s-LPS) of the outer membrane of smooth *Brucella* species (Bundle et al. 1987, Cherwonogrodzky et al. 1987). Certain biovars of *Brucella* have a

predominance of the A polysaccharide antigen. Examples include *B. abortus* biovar 1, and *B. suis* biovars 1, 2 and 3. Other biovars possess a predominance of M polysaccharide; examples include *B. melitensis* biovar 1 (Wilson and Miles 1932). *B. suis* 4 shows an equal proportion of both A and M antigens (Alton et al. 1975, Wilson, and Miles 1975). Thus, *B. suis* 3-vaccinated reindeer should be discriminated from *B. suis* 4-infected reindeer based on the presence or absence of antibodies directed against the M polysaccharide antigen. An indirect enzyme-linked immunosorbent assay (ELISA) was developed to detect reindeer antibodies specific for both A and M antigens. In addition to providing a means of discrimination between vaccinated and infected reindeer, this test would be highly sensitive in detecting reindeer with exposure to *Brucella.*

I tested the following hypotheses: (1) The indirect ELISA based on A antigen discriminates between reindeer with no previous exposure to *B. suis.* and those that have been exposed (this prior exposure may be due to either vaccination with *B. suis* 3 vaccine or infection with *B. suis* 4, and (2) The ELISA will discriminate between *B. suis* 4-infected and *B. suis* 3-vaccinated reindeer based on a differential antibody response to the M antigen.

METHODS

Production of Immunoglobulin G-specific Rabbit Anti-reindeer Serum

Immunoglobulin G-specific rabbit anti-reindeer serum was produced for the purpose of detecting reindeer IgG in the indirect ELISA. Serum protein in 100 ml of reindeer serum was precipitated with 27g of ammonium sulfate. The solution was stirred at 4°C for 12 h, centrifuged and the resulting pellet resuspended with sonication in 10S TEAN buffer consisting of 0.05M Tris HCl, 0.001M EDTA, 0.003M NaN_3, and 0.2M

NaCl. The sonicate was dialyzed in 10S TEAN buffer and desalted in Bio-Gel P-6DG[1] to remove remaining ammonium sulfate. Serum IgG was purified from the solution with DEAE Affi-Gel Blue[2]. First, the solution was dialyzed against a starting buffer of 0.02M Tris-HCl, pH 8.0, 0.028M NaCl, and 0.02% NaN_3. The gel was washed in 10 bed volumes of starting buffer, transferred to a column and eluted with 3 bed volumes of starting buffer prior to applying the desalted serum protein solution. After applying the solution, the column was again eluted with 3 bed volumes of starting buffer. Eluted fractions were collected in a Gilson FC-80 Micro-Fractionator[3]. Spectrophotometric absorbance values for the fractions were read at 280 nm. Fractions with an optical density reading of > 0.5 were saved and pooled. The eluted IgG was concentrated by suspending the solution in dialysis tubing in a vacuum flask at 4°C, until an optical density of 2 was reached. Azide was removed from the solution by dialysis. IgG purity was checked by sodium dodecyl sufate-polyacrylamide gel electrophoresis (SDS-PAGE) using a procedure described by Laemmli (1970).

The IgG solution was emulsified in Freunds complete adjuvant[4] in a 1:1 ratio, using a double-hubbed coupler and 2 Luer Lock syringes as described by Berlin and McKinney (1958). One milliliter of the resulting vaccine was injected into 6 adult Rex rabbits, with one-half given sub-cutaneously, and one-half given intramuscularly into the triceps femoris. The rabbits were re-inoculated 5 weeks later in the same manner. One week after the second inoculation, the rabbits were anesthetized with ketamine (35mg/kg) and xylazine (5mg/kg) and bled by cardiac puncture. The sera derived from these rabbits was examined in an ELISA against a commercially prepared rabbit anti-reindeer serum[5]

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1 Biorad Laboratories, 220 Wright Ave. Richmond, CA 94804.
2 Biorad Laboratories, 220 Wright Ave. Richmond, CA 94804.
3 Gilson Medical Electronics, Inc., Box 27, 3000 W Beltline Hwy, Middleton, WI 53562.
4 Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178.
5 Kirkegaard & Perry Laboratories Inc., Gaithersburg, MA.

for anti-reindeer IgG activity. All experimental procedures using live animals were approved by an independent animal welfare committee at the University of Alaska Fairbanks (UAF).

ELISA procedure

An indirect ELISA procedure developed by Douglas et al. (1984) was modified for detection of antibodies directed against both A and M *B. suis* epitopes. M and A-dominant antigens used in this test were acetone-killed whole cell preparations of *Brucella melitensis* strain 16M and *Brucella abortus* strain 119-3, (provided by B.L. Deyoe, U.S. Department of Agriculture, Ames, Iowa). Suspensions of each antigen were prepared in 0.01M ammonium acetate carbonate coating buffer (pH 8.2) at a concentration corresponding to a spectrophotometric optical density (OD) of 0.05 at 420 nm. Fifty microliters of antigen suspension was placed in wells of Immunolon 2 U plates[6] and allowed to dry overnight in an incubator at 37°C. *B. melitensis* antigen was placed in the 1st, 2nd, 5th and 6th plate rows and *B. abortus* antigen was placed in the 3rd, 4th, 7th and 8th rows. This arrangement allowed for duplicates of each reindeer sample to be tested on both antigens in a sample configuration as suggested by Stemshorn et al. (1983).

Nonspecific protein binding was blocked by the addition of 10% powdered skim milk[7] to the working buffer, a phosphate-buffered saline containing 0.1% Tween, of pH 7.4. Common determinants of A and M epitopes were blocked by an incubation with a monoclonal antibody, C-1, developed by Douglas and Palmer (1988). Plates were incubated with 100 µl of C-1 at a dilution of 1:1,000 at 37°C for 45 min prior to the addition of test sera from reindeer. One-hundred microliters of test serum diluted at 1:5,000 were added to each well and incubated for 1 h. This was followed by a 1 h

6 Dynatech Laboratories, Inc., Alexandria, VA.
7 Carnation Co., Los Angeles, CA 90036.

incubation with rabbit anti-reindeer IgG at a dilution of 1:20,000. Bound rabbit antibody was detected by a commercially prepared peroxidase conjugated IgG fraction goat anti-rabbit IgG[8], which was incubated for 45 min at a dilution of 1:8,000.

An ortho-phenylenediamine (OPD)[9] chromogen was used to indicate bound conjugate. This was prepared as a 6 mg/ml solution in citrate buffer (pH 5.2) of which 50 µl was added to each well. Plates were incubated for 15-20 min prior to termination of the peroxidase-chromogen reaction by the addition of 50 µl of 2.5 N sulfuric acid. Inter-plate variability was reduced with the use of a timing protocol that targeted consistent color development in positive control sera (Wright et al. 1985). Optical density (OD) of control sera obtained at 4 min and 414 nm were used to calculate the time of test termination. This "target" time was calculated by a micro-computer and ELISA program, where the computer was interfaced with a Titertek Multiscan[10] ELISA reader. The ELISA program used was "ELISA" version 1.1, written by W. Kelly, ADRI, Agriculture Canada (Cherwonogrodzky et al. 1990). The peroxidase-chromogen reaction was terminated by the addition of 2.5 N sulfuric acid, and color production was read at 492 nm.

Each sample was tested in duplicate on both A and M antigens. Percent co-variances were calculated for each duplicate. Where a significant difference occurred between duplicates on one or both antigens, the sample was re-tested.

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8 Cappel Laboratories, Organon Teknika Corp., 1230 Wilson Dr., West Chester, PA 19380
9 Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178.
10 Flow Laboratories, 7655 Old Springhouse Rd., McLean, VA 22102.

Control Sera

Serum known to contain anti-*B. suis* antibody was derived from a reindeer that was challenged with 3 x 10^7 CFU of *B. suis* 4. This serum is hereafter referred to as a positive control. *B. suis* 4 was isolated from this animal at necropsy. The control sample was taken 2 months post-challenge and produced agglutination: (1) On the buffered *Brucella* antigen (BBA) test, (2) At a serum dilution of 1:400 on the standard plate (SP) test, and (3) At a serum dilution of 1:400 on the Rivanol (Riv) test. This sample produced a reading of approximately 1.5 OD on both A and M antigens ELISA. Serum with no anti-*B. suis* antibody was taken from a reindeer with no previous exposure to *Brucella*, and is hereafter referred to as a negative control. Values of approximately 0.05 OD were recorded on both ELISA antigens. Reindeer serum for a vaccine control was taken from an animal 2 months after vaccination with heat-killed *Brucella suis* biovar 3 in Freunds incomplete adjuvant (FIA). This sample approximated 0.4 OD on A antigen ELISA and 0.7 OD on M antigen ELISA and produced agglutination: (1) On the buffered *Brucella* antigen (BBA) test, (2) At a serum dilution of 1:400 on the SP test, and (3) At a serum dilution of 1:400 on the Riv test.

Test Sera

A) Negative sera

Blood was collected from reindeer that had no possibility of exposure to *B. suis* 4. These animals had been housed at the Large Animal Research Station and reindeer holding facility at the UAF. Thirty sera were selected randomly from 150 possible samples.

B) Positive sera from infected reindeer

Thirty samples from infected reindeer were selected randomly from 80 possible samples. *B. suis* 4 had been isolated from all these animals. These animals represented

both laboratory and field-infected reindeer, including 12 calves. The samples chosen were expected to represent both chronically and acutely infected reindeer.

Nineteen of 30 sera were from Seward-Peninsula reindeer with brucellosis lesions from which *B. suis* 4 was isolated. These lesions included swollen joints or associated bursa (42%), testicular abscesses (42%), necropurulent lymph nodes (10%), and vaginal discharges (5%). Abscesses and necropurulent brucellosis lesions traditionally have been regarded as chronic manifestations of brucellosis in reindeer (Nikolaevskii 1961, Zabrodin et al. 1980, Dieterich 1981). One of these infected reindeer showed no evidence of previous exposure based on agglutination tests.

Eleven additional samples were obtained from reindeer infected in laboratory vaccine trials. The reindeer had been sampled sequentially over 4 months following challenge. A sample for testing was selected randomly from each sequence. Two 1-month-old infected calves of challenged females also were tested. The 12 samples from these experimental reindeer were considered to be from recently infected animals.

C) Sera from vaccinated reindeer

Samples from vaccinated reindeer were derived from two sources. Eleven samples were taken from reindeer in vaccine-efficacy experiments (Chapters 1 and 3) 2 months after vaccination with a killed *B. suis* 3 vaccine.

Other samples from vaccinated reindeer were collected from members of a free-ranging herd. In November 1988, 100 reindeer in the Shaktoolik reindeer herd were vaccinated with *B. suis* 3 vaccine. This herd of approximately 1,200 animals was considered relatively free of brucellosis. One-hundred reindeer in this herd had been tested in June 1988 by UAF researchers. No serologic evidence of exposure to *B. suis* 4 was detected by means of BBA, SP or Riv. The range of the Shaktoolik herd overlapped with migratory routes of the Western Arctic caribou herd. This caribou herd showed an

18% prevalence of serum antibodies of brucellosis during the years 1986-1989, based on samples taken from 44 animals (R. Zarnke, pers. comm.) There were possibilities for transmission of brucellosis from infected migrating caribou to this "clean" herd, but no brucellosis lesions were observed.

The 100 animals vaccinated with *B. suis* 3 included 50 adult females, 25 6-month-old male calves, and 25 6-month-old female calves. These reindeer, and 16 additional control reindeer, were ear-tagged and bled at the time of vaccination in November 1988, and bled again in June 1989 ($n = 76$), November 1989 ($n = 70$) and June 1990 ($n = 46$). Thirty-three additional reindeer were bled in these successive handlings to monitor the background prevalence of brucellosis.

D) Serial samples from both vaccinated and challenged reindeer.

The normal course of antibody responses to A and M antigens in reindeer following vaccination or infection was further investigated by testing serial samples from 11 reindeer used in vaccine-efficacy trials at UAF (Chapter 1, Chapter 3). Serial samples from 4 *Rangifer*, which were vaccinated with *B. suis* 3 and monitored for 18 months following vaccination, were tested with ELISA. Serum samples derived from 7 reindeer vaccinated with killed *B. suis* 3 vaccine, and 2 months later challenge with *B. suis* 4, also were tested. Three of the 7 vaccinates and 6 controls had become infected with *B. suis* 4 following challenge. Sequential samples spanning the 6 months from the time of vaccination to necropsy were tested for all animals in each group.

Experimental Design

I tested the hypothesis that the A antigen-based ELISA discriminates between reindeer that previously have been exposed to *B. suis*, and those with no previous exposure, by comparing values of spectrophotometric absorbance obtained with samples from these two groups of animals. In this experiment, exposure to *B. suis* included

vaccination with *B. suis* 3 or infection with *B. suis* 4. I tested 30 "negative" and 177 "positive" samples on the A-antigen ELISA, where the "positive" group was composed of: (1) 30 samples from infected reindeer, (2) 11 samples from reindeer sampled 2 months post-vaccination, and (3) 76 samples from reindeer sampled 7 months post-vaccination. Discriminant function analysis (DFA) (Johnson and Wichern 1988) was used to classify negative and positive sera using values of spectrophotometric absorbance obtained on the A antigen. Prior probabilities used in the DFA were based on sample size. I also compared results from the A antigen-based ELISA with results from standard agglutination tests including the buffered *Brucella* antigen, standard plate, and Rivanol tests (U.S. Department of Agriculture not dated, b, c).

I hypothesized that the ELISA discriminates between *B. suis* 4-infected and *B. suis* 3-vaccinated reindeer based on a differential antibody response to the M antigen. This hypothesis was tested by comparing spectrophotometric absorbance values from vaccinated and infected reindeer obtained on the M antigen ELISA. DFA was used to classify 87 vaccinated reindeer sampled 2 and 7 months post-vaccination and 30 infected reindeer using M antigen ODs. The groups of reindeer vaccinated at 2 and 7 months were combined for this analysis to form a representative group of vaccinated animals with the largest possible sample size. Prior probabilities used in this analysis again were based on sample size.

I also assessed an alternative means of discrimination, based on the percentage difference in spectrophotometric absorbance values obtained on A and M antigens. DFA was used to classify 87 vaccinated reindeer sampled 2 and 7 months post-vaccination and sera from 30 infected reindeer using the calculated percentage difference [% difference = (A - M)/A x 100)], where A = OD readings from the A-antigen ELISA.

Serial blood samples were obtained from reindeer after vaccination with *B. suis* 3 or infection with *B. suis* 4. Sera were tested to evaluate the changes in A and M-specific antibody responses over time. Polynomial regressions (5th order) were fitted to geometric means for OD readings from ELISA based on both A and M antigens; curves were fit as in Chapter 1. Partial *F*-tests were used to determine the number of parameters in these equations (Zar 1984). Autocorrelations in regression analyses were minimized by the selection of an optimal sampling interval. Weighted regression analysis was used where samples were missing from a series. Mean weighting factors in a series were never < 94% of full weighting. Nonlinear regressions, modifications of a half-normal distribution ($Y = ae^{bx}$), were fitted to mean percentage differences as measured over time. Regression lines modeling A and M responses in vaccinated and control reindeer were compared using the procedure of Zar (1984). Similarly, comparisons were made between regression lines fitted to percentage differences in vaccinated reindeer that were infected with *B. suis* 4 and those that were not infected.

RESULTS

Serologic Determination of Nonexposed, Vaccinated, and Infected Reindeer

Reindeer that had not been exposed to *B. suis* 4 could be differentiated from vaccinated and infected reindeer based on values of spectrophotometric absorbance obtained in the A antigen-based ELISA. Both vaccinated and infected reindeer had a high level of antibody against this antigen as measured by the ELISA (Table 2.1). This is in contrast to data for unexposed animals, which showed negligible levels of antibody to the A antigen (Figure 2.1).

ELISA determinations for the vaccinated group was composed of samples taken from 11 reindeer sampled 2 months post-vaccination, and 76 reindeer sampled 7 months post-vaccination. No overlap occurred in A antigen OD readings between the "positive"

TABLE 2.1.
Spectrophotometric absorbance values obtained on A and M-dominant antigens and percentage difference statistic [(A - M)/A x 100)] for 30 negative reindeer, 30 infected reindeer, and reindeer sampled at 2, 7, 12, and 19 months post-vaccination (P.V.) with *B. suis* 3 vaccine. Group sizes for the vaccinated reindeer were 11, 76, 70 and 46, respectively. Means, standard deviations and ranges are presented.

SAMPLE TYPE	A MEAN	SD	RANGE
NEGATIVE	0.04	0.02	(0.01-0.09)
INFECTED	1.56	0.21	(1.14-2.00)
2 MO P.V.	1.42	0.70	(1.20-1.58)
7 MO P.V.	1.52	0.27	(0.92-2.18)
12 MO P.V.	1.21	0.17	(0.65-1.44)
19 MO P.V.	1.22	0.20	(0.62-1.65)

SAMPLE TYPE	M MEAN	SD	RANGE
NEGATIVE	0.07	0.07	(0.02-0.37)
INFECTED	1.50	0.26	(0.77-2.00)
2 MO P.V.	0.61	0.32	(0.23-0.86)
7 MO P.V.	1.23	0.27	(0.58-1.78)
12 MO P.V.	1.04	0.20	(0.54-1.33)
19 MO P.V.	0.97	0.20	(0.33-1.36)

SAMPLE TYPE	%DIFF MEAN	SD	RANGE
NEGATIVE	NA		
INFECTED	3.9	11.0	(-34.4-31.9)
2 MO P.V.	57.5	14.3	(37.9-80.7)
7 MO P.V.	19.6	9.6	(5.4-48.8)
12 MO P.V.	14.3	9.8	(-3.1-40.4)
19 MO P.V.	21.1	10.0	(1.7-64.0)

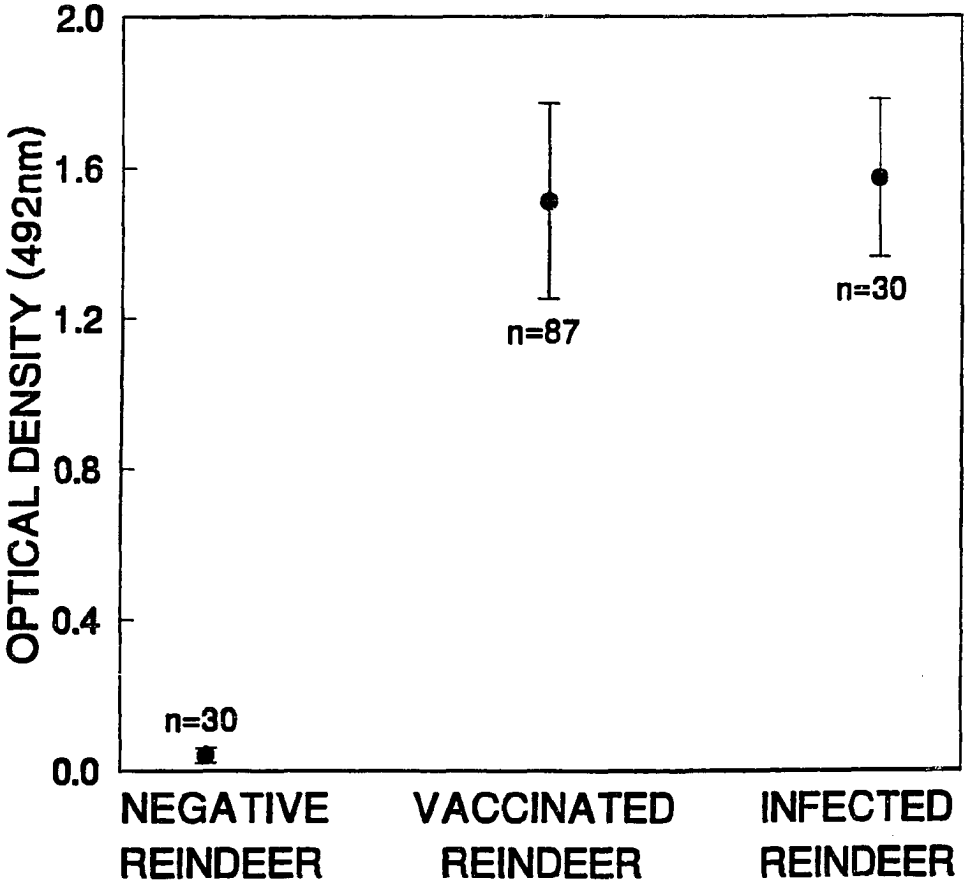

FIGURE 2.1.
ELISA optical density readings on A-dominant antigen. Means for three groups of negative, *B. suis* 3-vaccinated and infected reindeer are presented with bars representing ±1 standard error. *B. suis* 3-vaccinated reindeer include 11 reindeer sampled 2 months after vaccination, and 76 reindeer sampled 7 months after vaccination.

samples from vaccinated and infected reindeer and the "negative" samples from reindeer with no exposure to *B. suis*. All 147 samples taken from these 2 groups of positive and negative reindeer were classified correctly by DFA using OD readings from A antigen-based ELISA.

Criteria for differentiating reindeer that had been exposed to *B. suis* from those which had not were determined from the results of this analysis. Sample spectrophotometric absorbance values of < 0.15 OD were designated "negative," and sample OD readings of > 0.30 were designated "positive." Intermediate samples were designated as "doubtful."

Results of A antigen-based ELISA from these groups of negative and positive (vaccinated or infected) reindeer were compared with results from agglutination tests (Table 2.2). The ELISA was most sensitive, identifying all vaccinated and infected reindeer as "positives," whereas other agglutination tests did not. The sensitivity of the ELISA was 100% in this sampling, the BBA test was 97% sensitive, the SP test was 96% sensitive, and the Riv test, was 91% sensitive.

I expected that vaccinated reindeer would show negligible antibody activity. This expectation was not supported (Table 2.1). The reindeer sampled at 2 months post-vaccination showed the lowest readings and greatest contrast to the infected group on the M antigen-based ELISA. The M antigen-based ELISA discriminated between vaccinated reindeer and infected reindeer (sampled at 2 and 7 months post-vaccination) with a specificity of 98% and a sensitivity of 30% using DFA.

A high level of discrimination between vaccinated and infected reindeer was accomplished with the calculation of a percentage difference in spectrophotometric absorbance values obtained on the A and M antigen-based ELISAs. I expected that the calculated percentage difference for infected reindeer would approximate 0, whereas

TABLE 2.2
Results of A-dominant antigen based ELISA as compared with buffered *Brucella* antigen (BBA), standard plate (SP), and rivanol (Riv) tests for groups of 30 negative reindeer, 30 infected reindeer, and 87 reindeer vaccinated with killed *B. suis* 3 vaccine. The sensitivity of each test in identifying reindeer with exposure to *Brucella* is also given.

TEST RESULT		NEG (30)	VAC (87)	INF (30)	SENSITIVITY
BBA	+	0	87	27	97%
	-	30	0	3	
SP	+	0	86	26	96%
	-	30	1	4	
Riv	+	0	82	25	91%
	-	30	5	5	
A-ELISA	+	0	87	30	100%
	-	30	0	0	

vaccinated reindeer would produce a much higher percentage difference. The mean percentage difference for infected reindeer was 3.9% (Table 2.1 and Figure 2.2), supporting the assumption that *B. suis* 4-infected reindeer would show equal antibody production against both A and M antigens. The mean percentage differences for reindeer sampled at 2, 7, 12, and 19 months post-vaccination were 57.5%, 19.6%, 14.3% and 21.1%, respectively (Table 2.1 and Figure 2.2), supporting the assumption that there would be a much greater difference in vaccinated than in infected groups. There was a significant difference ($P < 0.001$) between the mean of the infected group and the means of these 4 vaccinated groups using the Mann-Whitney U-test [Z = -4.8 (2 mo), -6.4 (7 mo), -4.5 (12 mo), -6.1 (19 mo)]. Frequency distributions of percentage differences for vaccinated and infected reindeer are skewed, but distinct (Figure 2.3). DFA using rank-transformed data discriminated between vaccinated and infected reindeer with a 92% specificity (correctly classified negatives) and an 80% sensitivity (correctly classified positives). A total of 89% of vaccinated and infected reindeer were classified correctly by DFA based on rank-transformed percentage difference.

A simulation was performed to determine if increasing sample sizes would improve the percentage of reindeer correctly classified by DFA. A sample size of 30 for both vaccinated and infected groups resulted in a 74% correct classification using percentage difference as the only variable. Seventy-eight percent of reindeer were classified correctly with groups of 50, and 78% of the animals were classified correctly with groups of 75 in this simulation. These results indicate that a sample size of 50 to 75 for the infected group of reindeer would have improved discrimination.

Time Series ELISA Results in Vaccinated and Infected Reindeer

The antibody response to the M antigen never reached the level of the response to the A antigen (Figure 2.4). Regression equations fitted to antibody responses to A and M

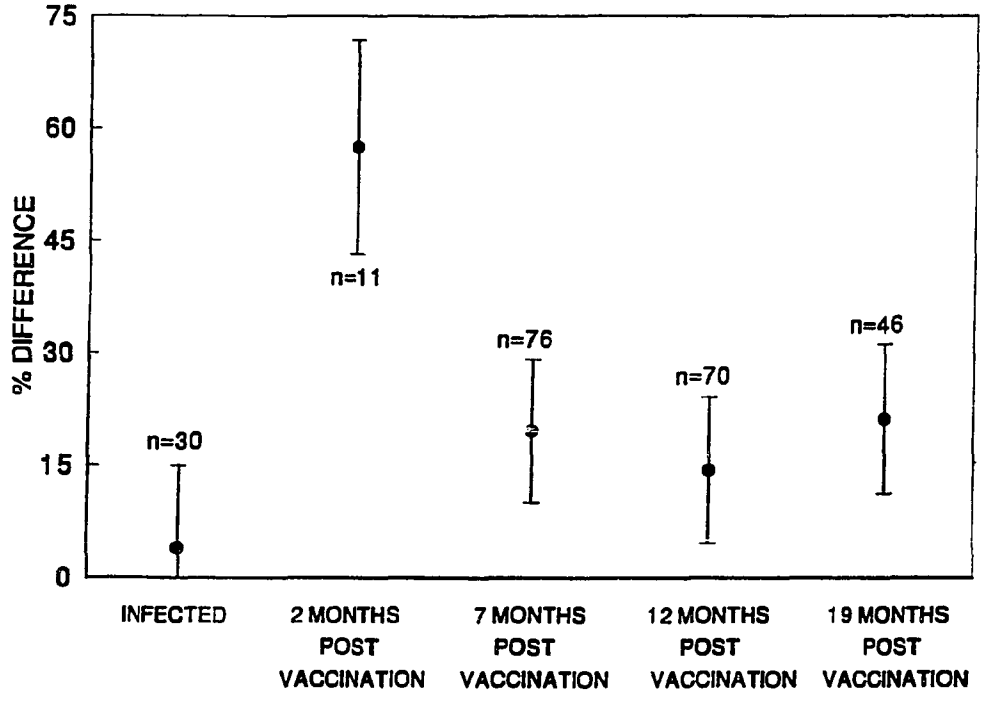

FIGURE 2.2.
Percentage differences in spectrophotometric absorbance values obtained on A and M-dominant antigens [(A - M)/A x 100)] for infected reindeer, and reindeer sampled at 2, 7, 12, and 19 months post-vaccination with *B. suis* 3 vaccine. Means for these groups are presented with bars representing ±1 standard errors.

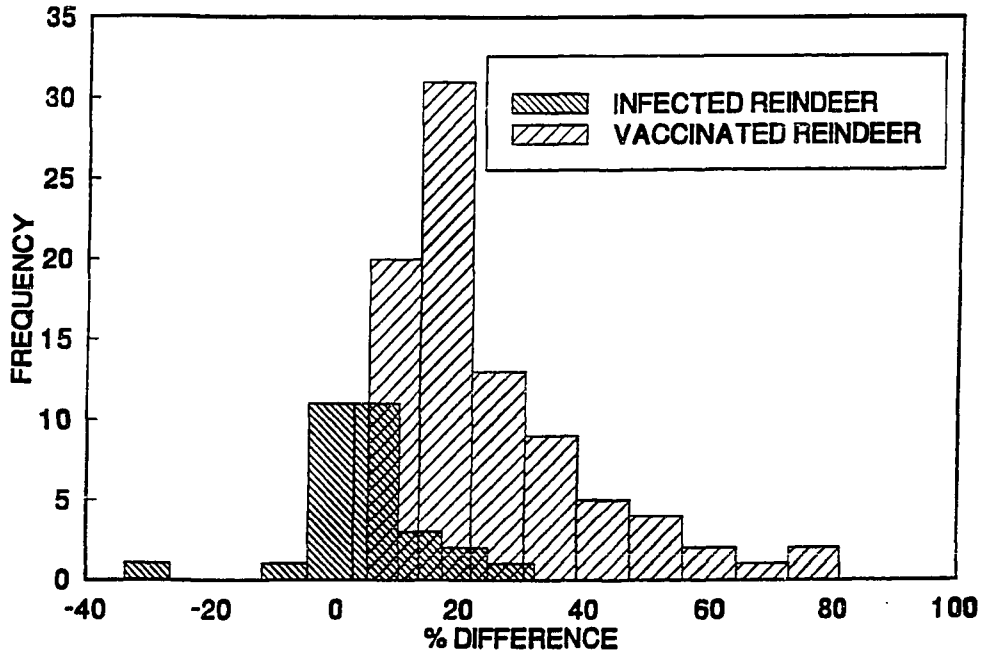

FIGURE 2.3.
Frequency histogram of percentage differences in spectrophotometric absorbance values obtained on A and M-dominant antigens [(A - M)/A x 100)] for 87 *B. suis* 3-vaccinated and 30 *B. suis* 4-infected reindeer. *B. suis* 3-vaccinated reindeer include 11 reindeer sampled 2 months post-vaccination and 76 reindeer sampled 7 months post-vaccination.

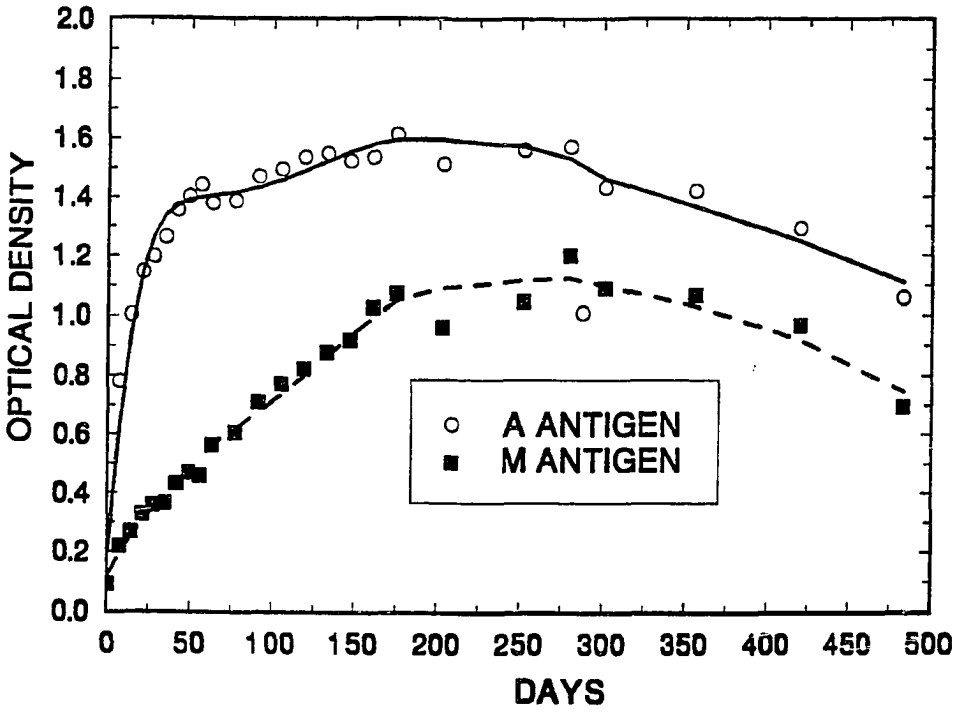

FIGURE 2.4.
Serologic response of 4 *Rangifer* to A and M-dominant antigens after vaccination with *B. suis* 3 vaccine. Reindeer were sampled sequentially for 18 months after vaccination. Results are presented as geometric means of A and M antigen ELISA spectrophotometric absorbance values obtained on A and M-dominant antigens along with weighted polynomial regressions.

$\hat{Y}$ (A antigen) = $0.456 + (0.316 \times 10^{-1})X - (0.326 \times 10^{-3})X^2 + (0.149 \times 10^{-5})X^3 - (0.306 \times 10^{-8})X^4 + (0.230 \times 10^{-11})X^5$.

$r^2 = 0.88$

$\hat{Y}$ (M antigen) = $0.146 + (0.697 \times 10^{-2})X - (0.300 \times 10^{-5})X^2 - (0.900 \times 10^{-7})X^3 + (0.271 \times 10^{-9})X^4 - (0.257 \times 10^{-12})X^5$.

$r^2 = 0.98$

antigens were significantly different ($F = 102.37, P < 0.001$). Percentage difference in antibody responses to the A and M antigens in 4 *Rangifer* monitored for 483 days post-vaccination showed a marked decline from 70% 28 days post-vaccination, to 16-41% 280 days later (Figure 2.5). A percentage difference of approximately 20% was maintained even while titers decline.

The other sample series was obtained from 7 reindeer challenged with virulent *B. suis* 4 at 63 days post-vaccination. Three of these challenged reindeer subsequently became infected with *B. suis* 4 (Chapter 3). I expected that the percentage difference for vaccinated reindeer that were challenged but not infected would remain above the 20% level, whereas the percent difference for vaccinated reindeer that were later infected would fall to approximately 0%. The percentage difference results from vaccinated reindeer that were challenged (Figures 2.6 and 2.7) support this assumption. When measured at 175 days, percentage differences for uninfected reindeer were above the 30% level in all but 1 animal, which was 12%. In contrast, percentage difference for vaccinated reindeer that became infected began to decline markedly at the time of challenge in 2 of 3 reindeer. Final samples taken from these 3 reindeer at 175 days post-vaccination and 112 days post-challenge approximated a 10% difference. Regression equations tracking percentage differences in infected and noninfected vaccinates were significantly different ($F = 22.64, P < 0.001$).

The A antibody response curve differed significantly from the M antibody curve for all vaccinated reindeer, regardless of infection status [$P < 0.001$; $F_{\text{(infected vaccinates)}} = 53.61$, $F_{\text{(uninfected vaccinates)}} = 36.08$]. All vaccinated reindeer showed a strong antibody response to the A antigen within 14 days of vaccination, with percent differences ranging from 24-78%. This was followed by a slowly increasing antibody response to the M antigen. The A and M antibody responses in infected reindeer

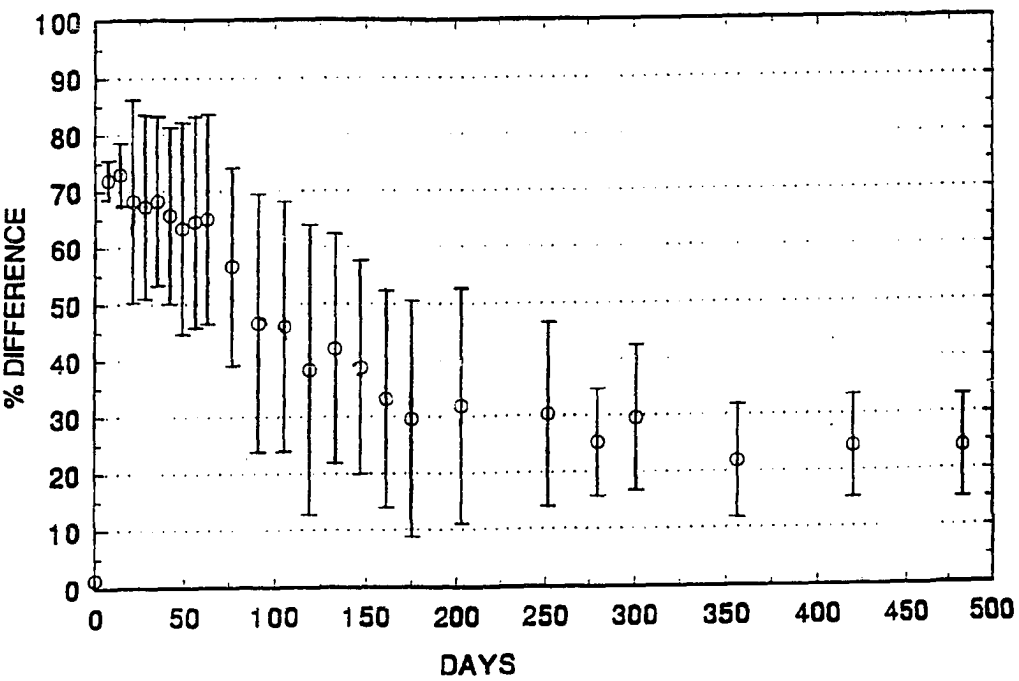

FIGURE 2.5.
Percentage differences in spectrophotometric absorbance values obtained on A and M-dominant antigens [(A - M)/A x 100)] for 4 *Rangifer* sampled sequentially for 18 months after vaccination with *B. suis* 3 vaccine. Means ±1 standard error are presented.

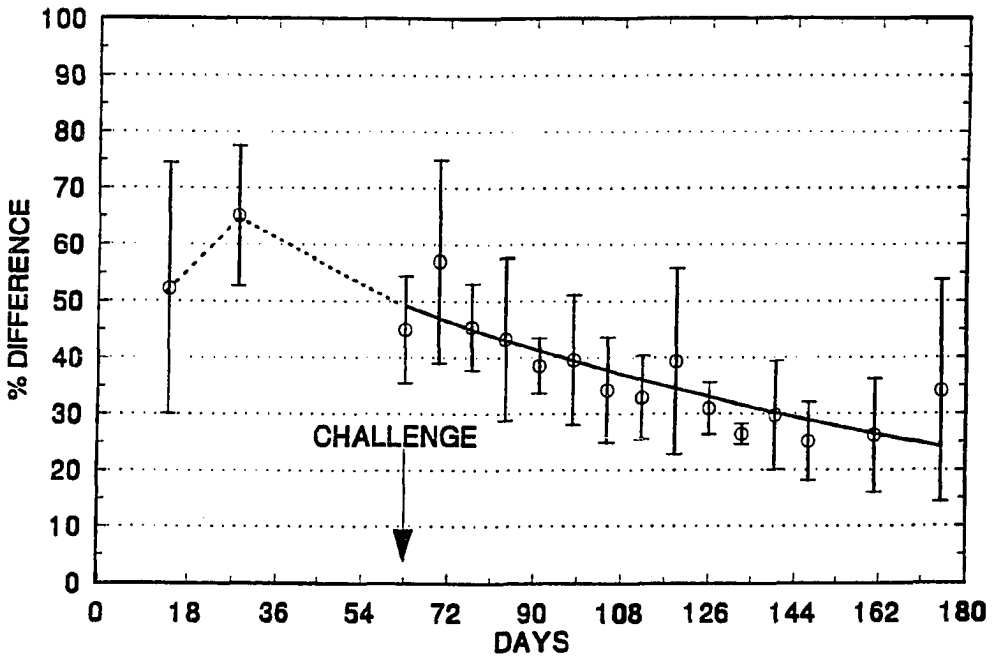

FIGURE 2.6.
Percentage differences in spectrophotometric absorbance values obtained on A and M-dominant antigens [(A - M)/A x 100)] for sequential samples obtained from 4 reindeer vaccinated with *B. suis* 3 vaccine and challenged 2 months later with virulent *B. suis* 4. Day 1 represents the day of vaccination. All four reindeer remained culture-negative for *B. suis* 4 following challenge. Means ± 1 standard error and non-linear regression are presented.

$\hat{Y} = 73.412 \times e^{-0.00636X}$ $r^2 = 0.71$

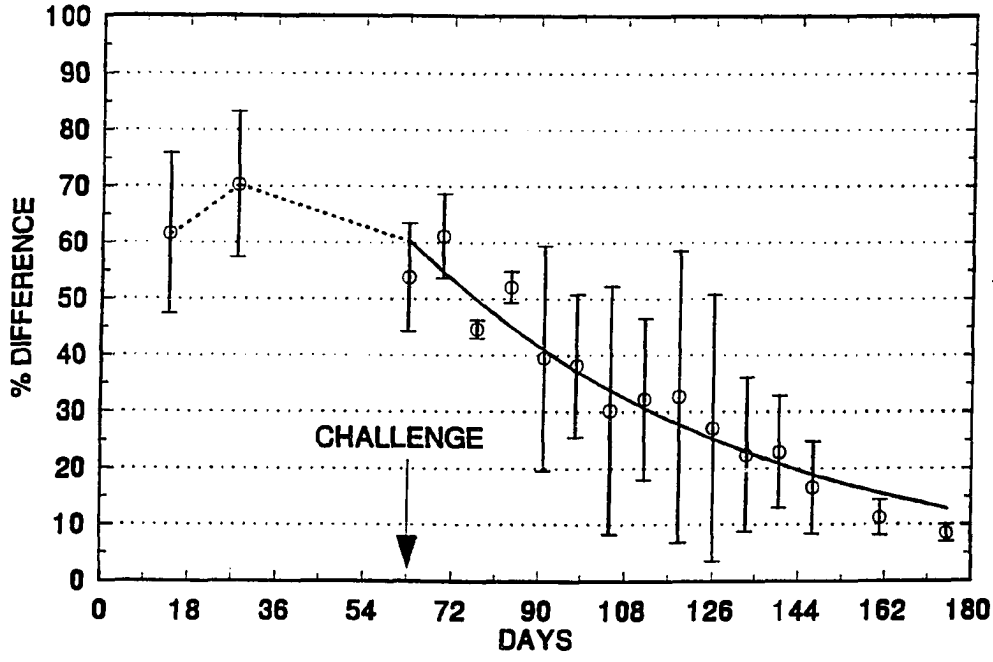

FIGURE 2.7.
Percentage differences in spectrophotometric absorbance values obtained on A and M-dominant antigens [(A - M)/A x 100)] for sequential samples obtained from 3 reindeer vaccinated with *B. suis* 3 vaccine and challenged 2 months later with virulent *B. suis* 4. Day 1 represents the day of vaccination. All three reindeer became infected with *B. suis* 4 following challenge. Means ± 1 standard error and non-linear regression are presented.

$\hat{Y} = 144.206 \times e^{-0.01384X}$ $r^2 = 0.92$

converged. Responses for *Brucella*-free reindeer remained distinct (Figures 2.8 and 2.9). This disparity was due to a difference in antibody responses to the M antigen following challenge (Figure 2.10). Regression equations tracking M antibody responses in the 2 groups were significantly different, ($F = 3.98$, $P < 0.02$), indicating that vaccinated reindeer, which later become infected, can be differentiated from noninfected vaccinates on the basis of the percentage difference.

Sequential samples from the 6 unvaccinated reindeer in the *B. suis* 4 challenge experiment also were tested on ELISA to track the antibody responses to A and M antigens following infection. I expected that these reindeer would develop both A and M-specific antibody responses at an equal rate, and that the percentage differences would remain static at approximately 0. This expectation was not met by data from the 6 nonvaccinated reindeer that were infected (Figures 2.11 and 2.12). Antibody response to the A antigen developed more rapidly than the response to the M antigen, creating a spike in percentage difference results shortly after challenge. This spike was as high as 50% in 4 of 6 reindeer 3 weeks after challenge, but fell near 0 within 7 to 9 weeks following challenge in 5 of 6 reindeer.

One of 6 nonvaccinated reindeer lagged behind others in developing an antibody response to infection. Antibody levels peaked much later and at a lower level than in other animals infected at the time of challenge. This animal exhibited a delayed antibody response to the M antigen in relation to the A response. The percentage difference in antibody responses to these antigens only had fallen to 21% 13 weeks post-challenge. The regression tracking the antibody response to the A antigen differed significantly from the regression for the antibody response to the M antigen in unvaccinated, infected reindeer ($F = 20.58$, $P = 0.001$).

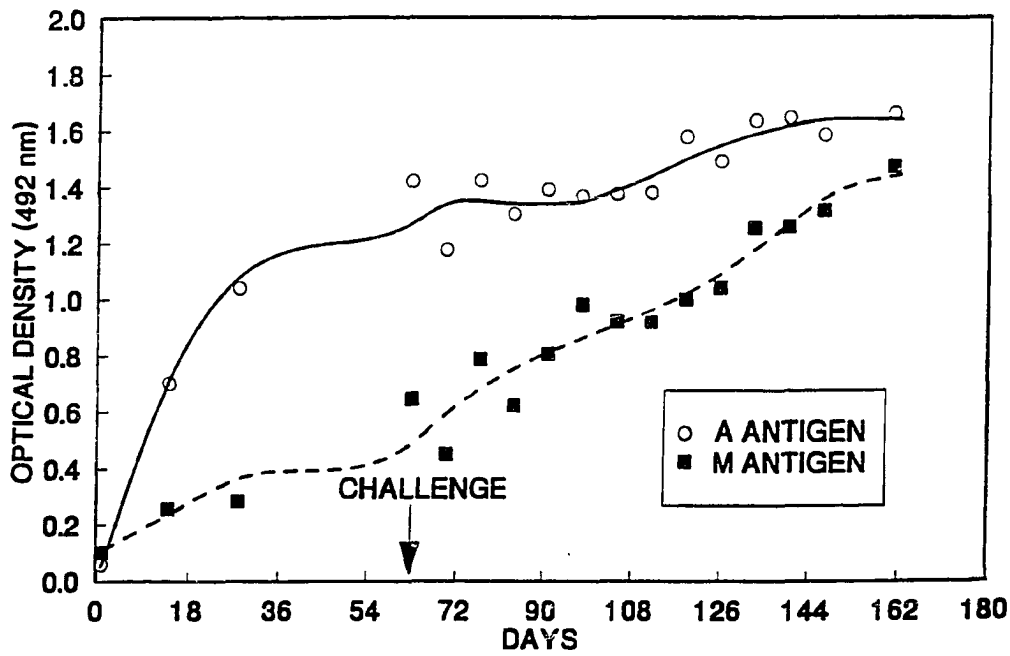

FIGURE 2.8.
Serologic response of 4 reindeer to A and M-dominant antigens after vaccination with *B. suis* 3 vaccine and subsequent challenge with virulent *B. suis* 4 two months later. Reindeer were sampled sequentially for 6 months from the date of vaccination (day 1). All four reindeer remained culture-negative for *B. suis* 4 following challenge. Results are presented as geometric means of ELISA spectrophotometric absorbance values obtained on A and M-dominant antigens along with polynomial regressions.

$\hat{Y}$ (A antigen) = $0.107 + (0.582 \times 10^{-1})X - (0.105 \times 10^{-2})X^2 + (0.954 \times 10^{-5})X^3 - (0.428 \times 10^{-7})X^4 + (0.756 \times 10^{-10})X^5$.

$r^2 = 0.93$

$\hat{Y}$ (M antigen) = $0.159 + (0.819 \times 10^{-2})X + (0.855 \times 10^{-4})X^2 - (0.237 \times 10^{-5})X^3 + (0.193 \times 10^{-7})X^4 - (0.528 \times 10^{-10})X^5$.

$r^2 = 0.90$

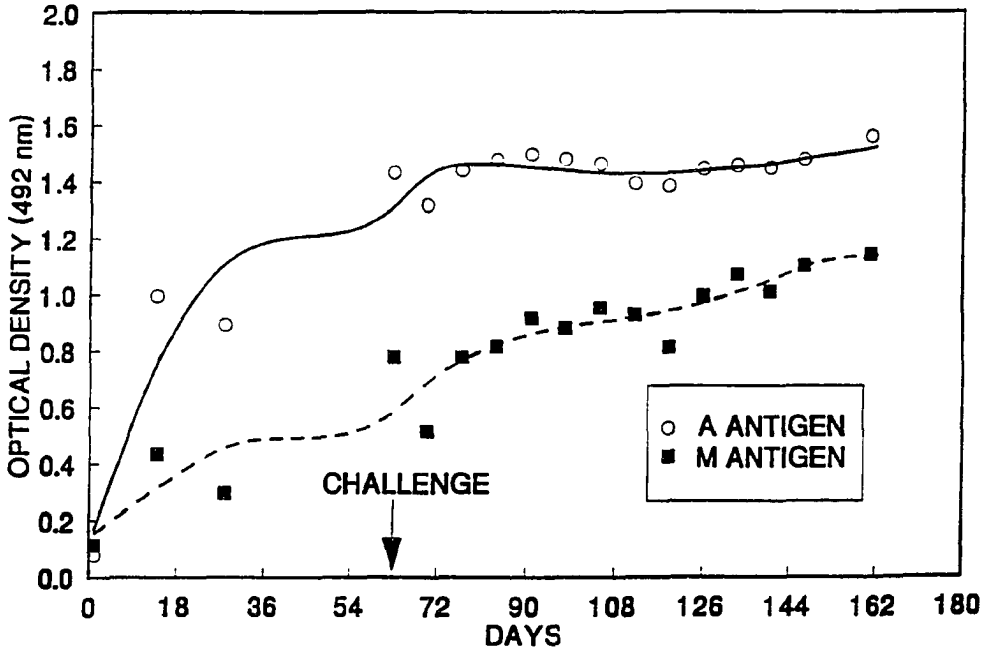

FIGURE 2.9.
Serologic response of 3 reindeer to A and M-dominant antigens after vaccination with *B. suis* 3 vaccine and subsequent challenge with virulent *B. suis* 4 two months later. Reindeer were sampled sequentially for 6 months from the date of vaccination (day 1). All three reindeer became infected with *B. suis* 4 following challenge. Results are presented as geometric means of ELISA spectrophotometric absorbance values obtained on A and M-dominant antigens along with polynomial regressions.

$\hat{Y}$ (A antigen) = $0.388x10^{-3} + (0.628x10^{-1})X - (0.110x10^{-2})X^2 + (0.836x10^{-5})X^3 - (0.235x10^{-7})X^4 + (0.852x10^{-11})X^5$.

$r^2 = 0.97$

$\hat{Y}$ (M antigen) = $0.117 + (0.470x10^{-2})X + (0.167x10^{-3})X^2 - (0.347x10^{-5})X^3 + (0.286x10^{-7})X^4 - (0.784x10^{-10})X^5$.

$r^2 = 0.96$

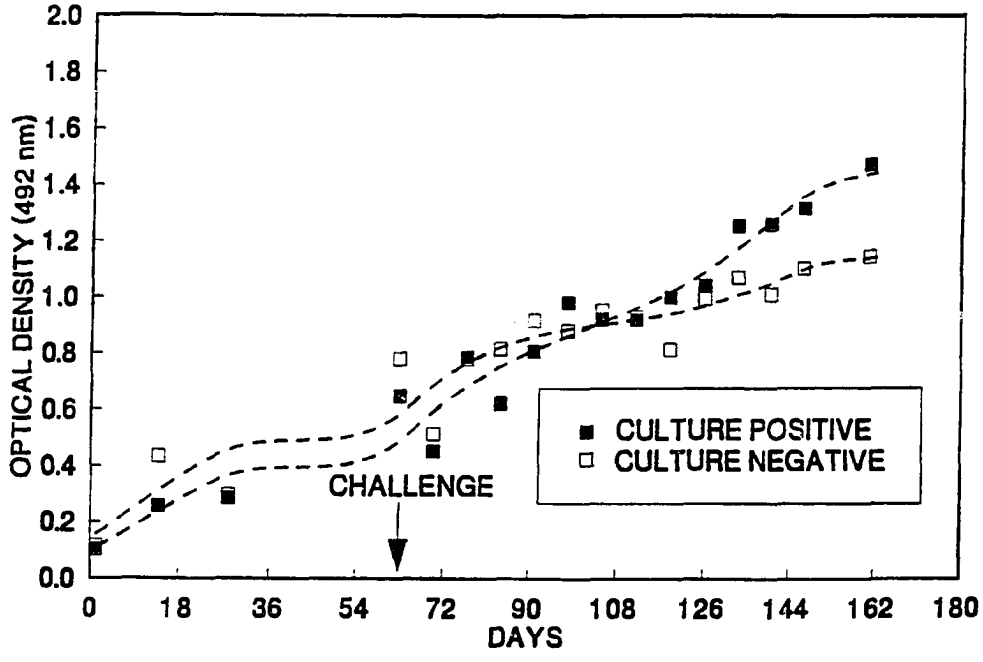

FIGURE 2.10.
Serologic response of 7 reindeer to M-dominant antigens after vaccination with *B. suis* 3 vaccine and subsequent challenge with virulent *B. suis* 4 two months later. Reindeer were sampled sequentially for 6 months from the date of vaccination (day 1). Four reindeer remained culture-negative for *B. suis* 4 following challenge, and three were infected. Results are presented as geometric means of ELISA spectrophotometric absorbance values obtained on M-dominant antigens along with polynomial regressions.

$\hat{Y}$ (culture-negatives) = $0.159 + (0.819 \times 10^{-2})X + (0.855 \times 10^{-4})X^2 - (0.237 \times 10^{-5})X^3 + (0.193 \times 10^{-7})X^4 - (0.528 \times 10^{-10})X^5$.

$r^2 = 0.90$

$\hat{Y}$ (culture-positives) = $0.117 + (0.470 \times 10^{-2})X + (0.167 \times 10^{-3})X^2 - (0.347 \times 10^{-5})X^3 + (0.286 \times 10^{-7})X^4 - (0.784 \times 10^{-10})X^5$.

$r^2 = 0.96$

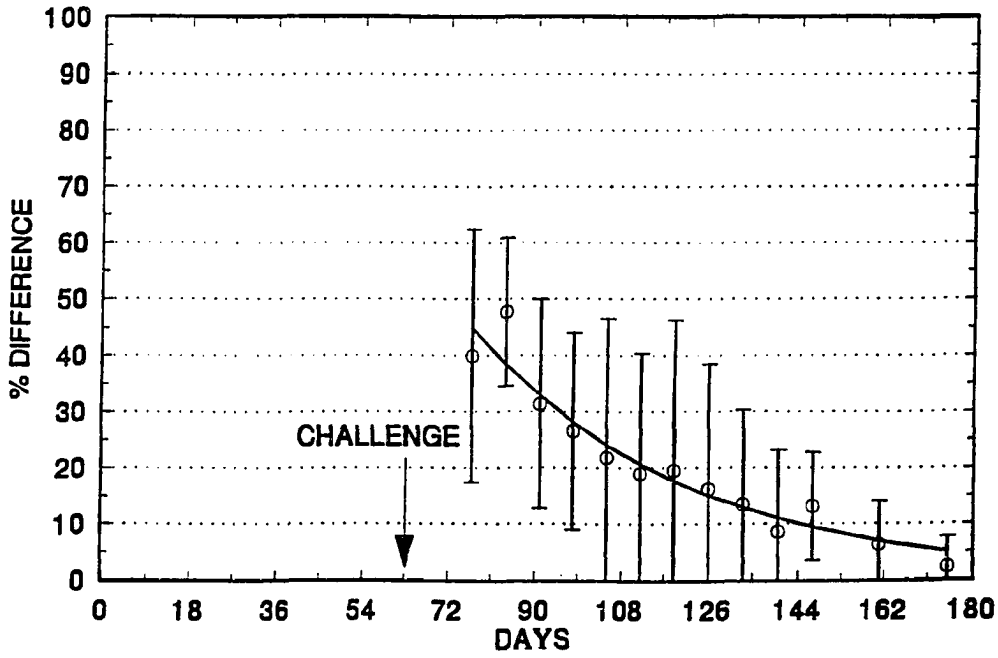

FIGURE 2.11.
Percentage differences in spectrophotometric absorbance values obtained on A and M-dominant antigens [(A - M)/A x 100)] for sequential samples obtained from 6 reindeer challenged with virulent *B. suis* 4. All 6 reindeer became infected with *B. suis* 4 following challenge. Means ±1 standard error are presented along with a weighted polynomial regression.

$\hat{Y} = 250.751 \times e^{-0.02233X}$ $r^2 = 0.92$

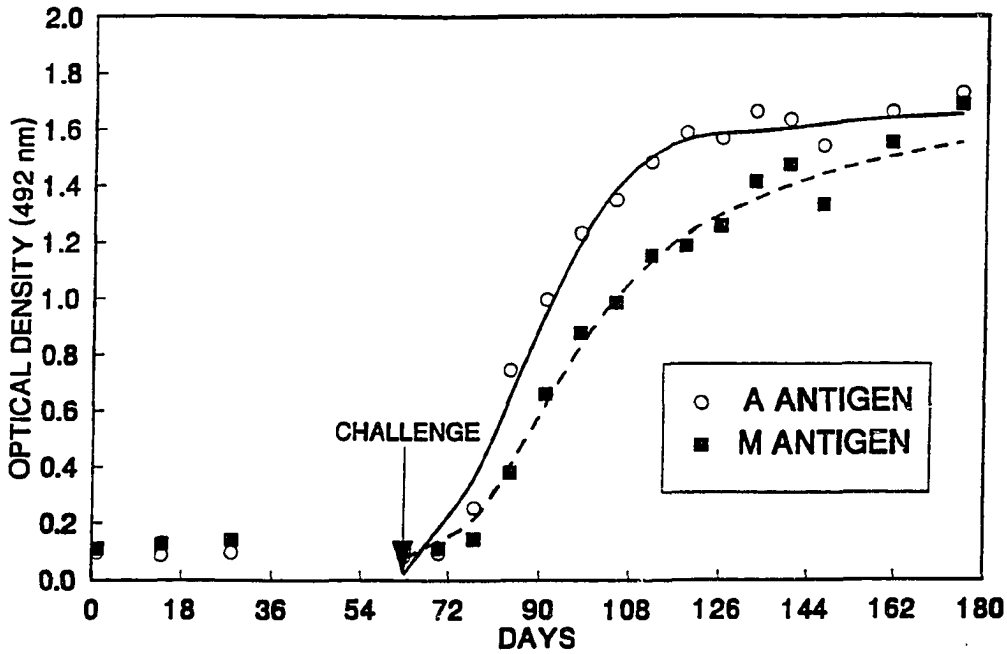

FIGURE 2.12.
Serologic response of 6 reindeer to A and M-dominant antigens after vaccination with challenge with virulent *B. suis* 4. Reindeer were sampled sequentially for 4 months from the date of challenge. All 6 reindeer became infected with *B. suis* 4 following challenge. Results are presented as geometric means of ELISA spectrophotometric absorbance values obtained on A and M-dominant antigens along with a weighted polynomial regression.

$\hat{Y}$ (A antigen) = $43.49 - 2.091\,X + (0.379\times10^{-1})X^2 - (0.324\times10^{-3})X^3 + (0.132\times10^{-5})X^4 - (0.210\times10^{-8})X^5$.

$r^2 = 0.99$

$\hat{Y}$ (M antigen) = $33.21 - 1.529\,X + (0.266\times10^{-1})X^2 - (0.219\times10^{-3})X^3 + (0.868\times10^{-6})X^4 - (0.133\times10^{-8})X^5$.

$r^2 = 0.99$

DISCUSSION

Use of ELISA for the Diagnosis of Brucellosis in Reindeer

The ELISA has a high degree of sensitivity and specificity in distinguishing between reindeer that have been exposed to *Brucella* and those with no previous exposure. This is true whether reindeer have been exposed by vaccination with *B. suis* 3 or by infection with *B. suis* 4. All 147 reindeer were classified correctly as "negative" or "positive" using A antigen-based spectrophotometric absorbance values by DFA. Consequently, I accept the first research hypothesis.

All ELISAs were conducted in parallel with standard agglutination tests. These results, which are presented here and elsewhere (Chapter 1, Chapter 3), indicate that the IgG-specific ELISA is the most sensitive test. The ELISA detects *B. suis*-specific antibody as early as either SP or BBA tests.

Discrimination Between Vaccinated and Infected Reindeer

Discrimination between vaccinated and infected reindeer based on a differential antibody response to the M antigen was not possible using available techniques. Reindeer vaccinated with *B. suis* 3 exhibited substantial M-specific antibody activity as measured in ELISA. Thus I reject the second hypothesis. *B. suis* 3 has been regarded as a typical A+M- serovar (Alton et al. 1975, Wilson and Miles 1975). Positive or negative signs indicate reactions with serum specific for A or M antigens. A recent study has shown that M polysaccharide constitutes a small fraction of the total O polysaccharide content of this biovar (Meikle et al. 1989). The high level of antibody against *B. melitensis* seen in reindeer vaccinated with *B. suis* 3 therefore is difficult to explain.

High OD readings obtained on the M antigen ELISA with sera from vaccinated reindeer could be explained by the binding of antibodies with specificities for antigens

other than the M antigen. This is a likely explanation considering the complex structure of the outer membrane of smooth *B. suis*.

The outer membrane of gram-negative bacteria contains lipoprotein, porins, phospholipids, and lipopolysaccharides (LPS) (Martin and Hancock 1990). The s-LPS of smooth gram-negative species consists of an O polysaccharide attached via a core oligosaccharide to lipid A, that anchors the molecule to the outer membrane (Cherwonogrodzky et al. 1990). The O polysaccharide, which contains the major antigenic epitopes of the s-LPS molecule and of the cell (Diaz et al. 1968, Cherwonogrodzky et al. 1990), projects outwards from the outer membrane surface. O polysaccharide on smooth *Brucella* spp. takes one of two forms: (1) An A antigen consisting of monomeric repeating units of 4,6-dideoxy-4-foramido-D-mannose with α 1,2 linkages (Caroff et al. 1984b) or (2) M antigen, identical to A antigen except that an α 1,3 glycosidic linkage replaces the α 1,2 linkage every 5th residue (Bundle et al. 1987). Research by Bundle et al. (1989) indicates that A-specific epitopes are α 1,2-linked pentasaccharides or larger oligosaccharides and M-specific epitopes are α 1,3-linked disaccharides with adjacent α 1,2 linkages. Epitopes consisting of linear portions of α 1,2-linked tri- or tetrasaccharides would constitute a large portion of both A and M polysaccharide molecules (Bundle et al. 1989).

The s-LPS of *Brucella* is highly heterogeneous in chemical structure and in immunogenicity between biovars and between LPS types (Caroff et al. 1984a, Dubray and Limet 1987, Palmer and Douglas 1989). The expression of s-LPS antigenic determinants in the heat-killed *B. suis* 3 vaccine and in the acetone-killed whole-cell preparations of *B. melitensis* and *B. abortus* could vary markedly. This is especially true where chemical or heat processing may have altered antigen structure. Many different types of antigenic determinants, other than those containing A and M-specific epitopes,

could be exposed in these whole-cell preparations. This could account for the high degree of antibody binding on *B. melitensis* seen with sera of vaccinated reindeer.

Improperly blocked epitopes common to both A and M polysaccharides could provide binding sites for antibody in sera of *B. suis* 3-vaccinated reindeer. As noted previously, common epitopes could constitute a large portion of the O polysaccharide molecule (Bundle et al. 1989). These may not have been sufficiently blocked by the monoclonal antibody C-1.

C-1 has been characterized as a monoclonal antibody that binds epitopes common to both A and M-sLPS, which are not present in *Yersinia enterocolitica* O:9 O polysaccharide (Palmer and Douglas 1989). The O polysaccharide chains of *enterocolitica* O:9 and *B. abortus* are identical (Bundle et al. 1984, Caroff et al. 1984a). This evidence suggests that C-1 may be specific for core components of s-LPS, rather than O polysaccharide components. C-1 therefore would not block O polysaccharide epitopes common to both A and M antigens in whole-cell preparations of *Brucella*. The presence of exposed common epitopes on *B. melitensis* polysaccharide could account for binding of antibodies from vaccinated reindeer.

The addition or substitution of additional monoclonal antibodies against common determinants of A and M antigens would likely improve ELISA discrimination of vaccinated and infected reindeer if common epitopes had not been sufficiently blocked. A combination of monoclonal antibodies may provide a better block of common A and M epitopes than a single antibody. Hewitt et al. (1982) increased the sensitivity of a competitive ELISA for the detection of tuberculosis in humans by the use of 2 or more inhibitive monoclonal antibodies. A combination of monoclonal antibodies with defined specificities (Douglas and Palmer 1988, Bundle et al. 1989) therefore may be needed to adequately block common determinants on the s-LPS molecule.

There is another explanation for the high level of antibody to *B. melitensis* antigens in sera of vaccinated reindeer. Perhaps antigens unrelated to O polysaccharide were exposed in the whole-cell antigen preparation. Lesser antigenic determinants such as outer-membrane lipids or proteins could provide binding sites for reindeer antibody regardless of the presence or absence of A and M antigens. This would result in "background" antibody binding on both types of whole-cell antigen. This "background" would be expected to gradually increase with time after exposure to *B. suis*.

This explanation is unlikely for two reasons. First, s-LPS-specific antibody interaction with s-LPS antigen probably obscures lesser antigenic determinants in whole-cell preparations (Nielsen and Wright 1984, Cloeckaert et al. 1990). Second, it is unlikely that sufficient antibodies specific for antigens other than s-LPS would be produced by vaccinated reindeer to account for high M antigen OD readings. Although antibodies are produced against antigens that are distinct from the s-LPS complex (Schurig et al. 1978), the primary antibody response of animals exposed to smooth *Brucella* species is directed against s-LPS (Diaz et al. 1968, Schurig et al. 1981, 1984, Moreno et al. 1987).

Discrimination between vaccinated and infected reindeer on the basis of the antibody response to the M antigen was not feasible. Nonetheless, discrimination was possible when the M-specific antibody level of vaccinated reindeer was assessed as a proportion of total antibody as detected by the A antigen-based ELISA. A percentage difference variable correctly classified 89% of 117 reindeer as either *B. suis* 4-infected or *B. suis* 3-vaccinated. The percentage difference was specific in discriminating between vaccinated and infected reindeer; false-positives totalled 8%. Nonetheless, the sensitivity was only moderate, with 80% of infected reindeer correctly identified. The ELISA

would be suitable for assessing the overall prevalence of brucellosis in herds of *B. suis* 3-vaccinated reindeer.

This test of discrimination, however, is not sensitive enough to apply to reindeer on an individual basis. The ability to identify infected individual reindeer in vaccinated herds is important for culling purposes and in selecting reindeer for shipment to *Brucella*-free areas. Ideally, a discriminatory test would identify 100% of infected animals. Unfortunately, ELISA criteria for classifying reindeer as "infected" cannot be established where all infected reindeer are included, without also including an unacceptably large proportion of negative, vaccinated reindeer. In addition, reindeer that have been recently infected (within a month or two of sampling) can not be differentiated from vaccinated reindeer.

Antibody Responses to A and M Antigens as Measured Over Time

ELISA results from reindeer sampled sequentially after exposure to *B. suis* showed that the antibody response to the A antigen predominated immediately after that event. In vaccinated reindeer, this was followed by a gradual increase in antibody to the M antigen, as detected in the ELISA. This increase occurred within the first 7 months after vaccination. Subsequently, an equilibrium was established where the antibody response to the M antigen was approximately 70-80% of the A-antibody response. Equilibrium was attained before antibody titers started to decline. The ELISA detection of antibodies specific for M antigen probably was not selective enough to exclude antibodies specific for determinants common to both A and M polysaccharide antigens. This would account for the gradual increase in measured M-antibody response in vaccinated reindeer.

The *B. melitensis* antigens responsible for the production of nonspecific agglutinins are likely to have been associated with the O polysaccharide molecule. This

suggestion is in accordance with those of Jones (1958), who sequentially bled rabbits up to 53 days after infection with *B. melitensis* to determine optimum-sampling time for the production of monospecific serum. M-Specific agglutinins (antibodies) predominated 6 days after inoculation. As time elapsed, the proportion of nonspecific, heterologous agglutinins in the sera increased. More recent research has shown that the O polysaccharide component of s-LPS is primarily responsible for the production of agglutinating antibodies (Diaz et al. 1968, Schurig et al. 1981). An increase in the production of common determinant-specific antibodies relative to the production of M-specific antibodies would explain the increase in heterologous agglutinins in rabbit sera as measured over time.

The antibody response to the M antigen developed more slowly than the response to the A antigen in *B. suis* 4-infected reindeer. This was unexpected. Recent research has indicated that in A+M+ biovars, such as *B. suis* 4, that A and M epitopes exist together on the same molecule rather than on two separate antigenic structures (Bundle et al. 1989, Meikle et al. 1989, Garin-Bastuji et al. 1990). Although the chemical structure of A and M epitopes has been identified (Bundle et al. 1989), their distribution on O polysaccharide chains has not been clarified. The lag in antibody response to the M antigen in infected reindeer indicates that M epitopes may occur more frequently on the core end or length of the O polysaccharide chain, rather than on the tip. There is, however, an alternative explanation. Past theories suggested that A and M antigens are separate molecular structures existing in equal proportions on the s-LPS of *B. suis* 4. Under this theory, a differential distribution of A and M common and M-specific epitopes along the M polysaccharide chain could account for the lag in antibody response to the M antigen in infected reindeer. The initial antibody response of infected reindeer may be directed against epitopes common to both A and M antigens, if these existed near

the tip of the M polysaccharide. Antibodies to determinants containing M-specific epitopes would appear later if these existed towards the core end of the O polysaccharide chain.

My hypotheses regarding the differential immunogenicity of epitopes as they are placed on the O polysaccharide chain are consistent with those proposed by Nielsen et al. (1989), and colleagues (Wright et al. 1990). They suggested that epitopes existing along the length of the O polysaccharide chain were only weakly immunogenic compared with the "tip" epitope. They also suggested that cattle would only produce antibodies to "length" epitopes when exposure was prolonged, as in infection.

The M-antibody response lags behind the A response in infected reindeer. The serologic response to these antigens, however, becomes equivalent. This indicates that equal quantities of both A and M epitopes are expressed in the *B. suis* 4 organism. Garin-Bastuji et al. (1990) noted results from latex-particle agglutination inhibition tests that suggested *B. suis* 4 showed a predominance of M antigen. This is contrary to the results of others who have reported equal A and M antigen expression in *B. suis* 4 (Dubray and Limet 1987, Douglas and Palmer 1988, Meikle et al. 1989, Palmer and Douglas 1989).

I documented that the ELISA will be a useful test in the diagnosis of brucellosis in reindeer. The ELISA is more sensitive than agglutination tests currently employed, and therefore will provide additional information used in adjunct to these tests. ELISA discrimination between *B. suis* 3-vaccinated reindeer and *B. suis* 4-infected reindeer is sufficient to allow assessment of the prevalence of brucellosis in vaccinated herds, but is not sufficient to identify infected individuals.

CHAPTER 3.

EFFICACY OF A KILLED *BRUCELLA SUIS* BIOVAR 3 VACCINE IN REINDEER CHALLENGE EXPOSED WITH *BRUCELLA SUIS* BIOVAR 4.

ABSTRACT

Seven female reindeer were vaccinated with killed *Brucella suis* biovar 3 vaccine. This group consisted of 5 pregnant adults and 2 8-month-old calves. These reindeer were challenged with 3.155×10^7 colony forming units of *Brucella suis* biovar 4 at 63 days post-vaccination. Five pregnant adults and 1 female calf served as experimental controls. *B. suis* 4 was isolated from 3 of 7 vaccinated reindeer (43%) at the time of necropsy. *B. suis* 4 was isolated from the aborted fetus of 1 of the infected vaccinates. Another infected vaccinate bore a healthy calf that was negative for *B. suis* 4 at necropsy. All control reindeer were infected and all 5 adults aborted. *B. suis* 4 was isolated from all 5 fetuses. The *B. suis* 3 vaccine provided significant protection against infection and abortion in reindeer challenged with *B. suis*.

INTRODUCTION

Brucellosis caused by *Brucella suis* biovar 4 is enzootic in Alaskan reindeer herds (Meyer 1966, Dieterich 1981). The main effect of the disease is on loss of productivity through abortion. Additionally, the disease causes orchitis, epidymitis and sterility in males (Golosov and Zabrodin 1959), and arthritis and bursitis with accompanying lameness in both sexes (Orloff 1963).

The serologic prevalence of brucellosis in certain reindeer herds in Alaska maybe as high as 20% (Dieterich 1981). A killed *B. suis* 4 vaccine (Dieterich et al. unpubl.) is currently used to control brucellosis in approximately 80% of Alaskan reindeer herds.

Serologic diagnosis of brucellosis in vaccinated reindeer is unfeasible. There are no detectable qualitative or quantitative differences in the antibody responses between vaccinated and naturally infected reindeer. My objective was to produce a vaccine that would allow serologic discrimination between vaccinated and infected reindeer. A killed *Brucella suis* strain 636 (biovar 3) was chosen as a vaccine. This biovar lacks the M polysaccharide antigen that is present in *B. suis* 4 (Wilson and Miles 1932). Antibodies directed against this antigen might be detected by means of a specifically designed enzyme-linked immunosorbent assay (ELISA). In this manner, vaccinated reindeer could be differentiated from naturally infected animals. I hypothesized that the *B. suis* 3 vaccine would engender sufficient immunity in reindeer to protect against challenge with virulent *B. suis* 4.

METHODS

Facilities

Reindeer were housed in special isolation facilities at the University of Alaska Fairbanks (UAF) from January 22 to May 25, 1989. These facilities met published Bio-Safety Level 3 requirements (Richardson and Barkley 1988). Five rooms of 10.5 m^2 held from 2 to 3 reindeer each. The containment area was accessible only through an air-lock system with "shower-in, shower-out" facilities. An additional air-lock provided access for the movement of equipment and supplies. Pass-through autoclaves and kill tanks for sewage provided for the removal of infected waste. Reduced air pressure was maintained in the facility and air was exhausted through absolute filters. Serologic and bacteriologic work was performed in isolation suites. Reindeer were bedded on wood

shavings that were replaced weekly. A commercial grain and pellet mixture[1] and water were offered *ad libitum* to reindeer. Experimental protocols for the study were approved by an independent animal welfare committee at UAF.

Vaccine Preparation

Brucella suis biovar 3 cells were produced in the following manner. Cells of *B. suis* strain 636 (Deyoe 1967) stored in a lyophilized state were reconstituted and inoculated onto a tryptose agar[2] plate. Cells grown on the plate were observed for colony purity and morphology and inoculated onto slants of potato infusion agar (PIA). Samples were incubated for 48-72 h after which they were tested for typical characteristics of *B. suis* 3 (Alton et al. 1975). A second set of PIA slants was inoculated and incubated for 24 h. Cultures from these slants were used to inoculate 2 Roux flasks containing tryptose agar. The flasks were incubated for 2-3 days after which cells were harvested and examined for viability on tryptose agar slants. Cultures from the Roux flasks were then used to seed media in a fermentation vessel. The production of cells in the fermenter vessel followed the procedure outlined in Alton et al.(1975) for batch culture on liquid media. Cells were incubated for 48 h using an initial agitation and aeration rate of 300-600 RPM and 6-14 l/min, respectively, for the first 24 h. In the following 24 h, agitation and aeration rates were kept at 600 RPM and 14 l/min. Cells were concentrated by centrifugation at 10,000 x g for 45 min. Viability was examined before and after this procedure. Cells were washed in saline, refrigerated overnight, resuspended in saline at 100mg/ml and heat-killed in a water-bath at 65°C for 1 h. Cell viability was examined again. Cells were then concentrated for storage by centrifugation and resuspended at 250 mg/ml in phosphate buffered saline (PBS).

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1 Quality Texture, Fisher Mills Inc., Seattle, WA.
2 Difco Laboratories Inc., Detroit, MI.

Cells of *B. suis* 3 then were emulsified in Freund's incomplete adjuvant (FIA)[3]. Twelve milliliters of cell concentrate were mixed with 388 ml of PBS. This suspension was emulsified in 200 ml of FIA in a blender vessel. The prepared vaccine contained 10 mg of cells per 2 cc vaccine dose. Vaccine was decanted into sterile multidose vials. All vaccine vials were examined for contaminants by culture on blook agar.

Experimental Procedure

Ten mature female reindeer and three 8-month-old female reindeer calves were brought into isolation facilities mid-January 1989. Mature reindeer had been with males during the previous September and October and were judged to be pregnant via a progesterone assay[4] (McEwan and Whitehead 1980, Ringberg and Aakvaag 1982) conducted in November. All calves were categorized as nonpregnant by this same assay.

There was no serologic evidence of *B. suis* exposure in any of these reindeer as determined by the standard plate (SP), Rivanol (Riv), buffered *Brucella* antigen (BBA)(U.S. Department of Agriculture not dated, b, c) and indirect ELISA tests (Chapter 2). *B. suis* 4 was not isolated from the blood of any reindeer. All reindeer were naive to challenge by *Brucella* organisms because all but one had originated from an isolated herd kept in double-fenced pens in a brucellosis-free area of Alaska. There had been no introductions of reindeer into the herd except for 2 reindeer that had been translocated from the Seward Peninsula 4 years previously. There was no serologic evidence of *B. suis* exposure in these 2 animals at the time of their introduction into the herd or any time thereafter.

Five adult female reindeer and 2 female calves were vaccinated sub-cutaneously in the right mid-cervical area with 2 ml of vaccine on 23 November 1988. Calves were

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3 Lee Laboratories, Inc. 1475 Highway 78, S.W., Grayson, GA 30221.
4 Veterinary Reference Laboratories, 411 N.E. 122nd Ave., Suite 140, Portland, OR 97230

approximately 8 months-of-age at the time of vaccination. Blood samples for serologic testing were taken every 2 weeks until mid-January at which time the reindeer were brought into isolation facilities. On 1 January 1989, these reindeer were challenged with 3.16×10^7 colony-forming units of *B. suis* biovar 4 instilled in the conjunctival sac. Six unvaccinated reindeer consisting of 5 pregnant females and 1 female calf were similarly challenged. The challenge strain had been isolated from a Seward Peninsula reindeer and had been passaged through lemmings (*Lemmus sibiricus*) to assure virulence. This challenge procedure had been utilized in previous experiments (Dieterich et al. 1980, 1981, unpubl., Dieterich and Morton 1987) and did not produce overwhelming infections in reindeer.

Blood samples were collected weekly for serologic testing and attempted bacterial isolation. Lesions of suspect *Brucella* etiology were cultured. Reindeer were observed at 12-h intervals after 1 March 1989. Aborted fetuses and calves that died were necropsied immediately. Placentas and vaginal swabs were collected from cows immediately after parturition or abortion. Vaginal swabs were taken from cows on a weekly basis until bacteria were isolated. Milk samples from cows and blood samples from calves were collected weekly for bacterial isolation and serology. In late May, approximately 1 month after calving, reindeer were euthanized and necropsied. Blood and tissue samples were collected for bacterial isolation.

B. suis 4 infection in reindeer at necropsy was assessed by the culture of the following tissues: right and left prefemoral, popliteal, prescapular, mandibular, parotid, suprapharyngeal, retropharyngeal, supramammary, mediastinal, mesenteric, bronchial, internal iliac and external iliac lymph nodes, liver, lung, spleen, kidney, muscle, and uterus. Aborted fetuses also were necropsied, and stomach contents, spleen, liver, lung, and both kidneys were cultured. All tissues were collected aseptically, flamed and

halved. The cut surface was minced with a scalpel. This was then inoculated onto both a TSA plate and a more selective media plate containing per liter: 25g tryptose broth, 20g of agar[5], 0.15ml of Tergitol 7, 25ml of Tween 40, 1.4 mg of ethyl violet, 1.44g of sodium lauryl sulfate, 1 vial of CNV[6], and 500mg of cylcoheximide[7]. Vaginal swabs, and swabs of milk and stomach contents were similarly inoculated on both media plates. Plates were incubated at 37°C and 5% carbon dioxide.

B. suis 4 cultures were identified by colony morphology, gram stain, agglutination with positive guinea-pig serum, absence of motility, culture on TSA plates containing the inhibitory dyes thionin 1:100,000 and fuschin 1:100,000 and the following biochemical tests: Hydrogen sulfide production, catalase and urease production (U.S. Department of Agriculture not dated, a).

Serology

All serologic tests were performed the Veterinary Services Laboratory at UAF. Standard procedures of the US Department of Agriculture were used for the SP, BBA, and Riv tests. An animal was considered positive for serologic tests if: (a) Agglutination occurred at a serum dilution of 1:25 on the SP, or (b) Agglutination occurred on the BBA, or (c) Incomplete agglutination occurred at a 1:25 dilution on the Rivanol test. Serologic tests used for milk were the *Brucella* ring test (BRT) (U.S. Department of Agriculture not dated, b) and whey-plate test (Cameron et al. 1956). The ELISA used was an indirect assay developed at the University of Hawaii (Douglas et al. 1984) and modified for use in reindeer (Chapter 2). Sera meeting or exceeding minimal titers hereafter are referred to as positive; all others are referred to as negative. Polynomial regression and curve fitting follow procedures outlined in Chapter 1.

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5 Bacto Agar, Difco Laboratories, Detroit, Mich.
6 Cholestimethate, nystatin, vancomycin, Difco Laboratories, Detroit Mich.
7 Actidione, Sigma Chemical Co., St. Louis, MO

Bacteriology

Bacteremia in reindeer was assessed by weekly blood culture. Five milliliters of blood was added to a trypticase soy agar (TSA)[8] slant bottle containing 20 ml of tryptose broth[9] with 1% sodium citrate. Agar slants were incubated at 37°C and 5% carbon dioxide for 5 weeks, with inspection for bacterial growth and shaking every 3 days. Bacterial growth occurring on slants was transferred to TSA plates for further evaluation. This process will be hereafter referred to as hemoculture.

RESULTS

Serology

Reindeer in both vaccinated and unvaccinated groups developed high levels of antibody in response to vaccination or infection (Figures 3.1, 3.2, and 3.3). All vaccinated animals had developed detectable levels of antibody, including positive BBA results, within 14 days of vaccination. Detectable levels of antibody were maintained in vaccinated reindeer through most of the experiment, with a marked rise in antibody levels following challenge with *B. suis* 4.

All control reindeer exhibited serologic evidence of exposure by 21 days post-challenge. The SP test was the first to detect a humoral response in control animals. BBA results were positive in these animals by 14-21 days after challenge, except for 1 reindeer that was not positive on the BBA until the 35th day after challenge.

Vaccinates and controls were positive on all serologic tests at the time of necropsy, except for 1 control reindeer that was negative on the rivanol test. Agglutination titers were generally lower in the control group of reindeer.

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8 Trypticase Soy Agar, Baltimore Biological Laboratories, Cockeysville, Md.
9 Tryptose Broth, Difco Laboratories, Detroit, Mich.

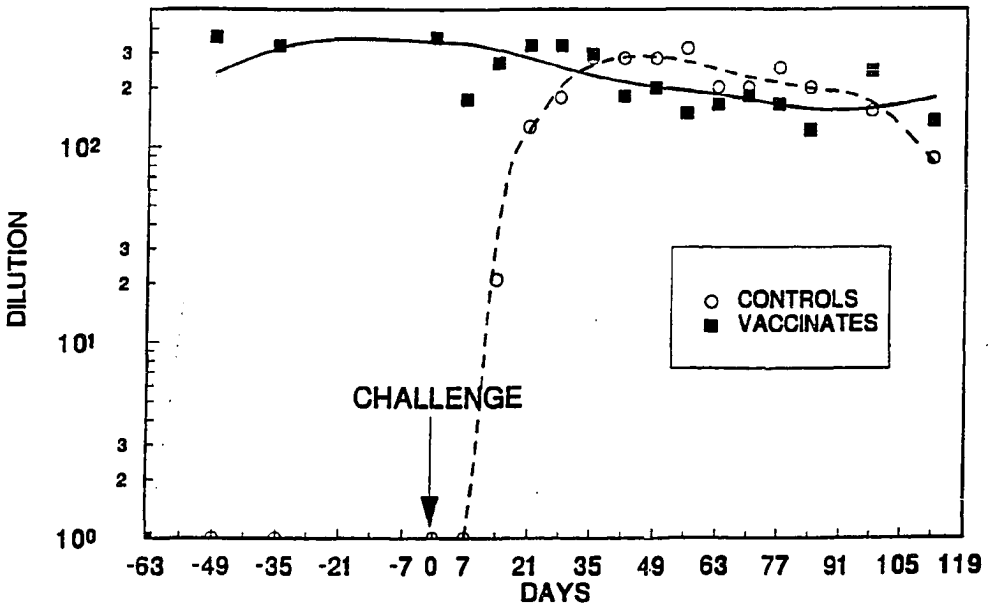

FIGURE 3.1.
Standard plate test results for 7 reindeer vaccinated with *B. suis* 3 vaccine, and 6 non-vaccinated control reindeer. Both groups were challenged with virulent *B. suis* 4 on day 0. Results are presented as geometric mean titers and weighted polynomial regressions.

$\hat{Y}$ (vaccinates) = $7.151 + 27.51\,X - 0.680\,X^2 + (0.705\times10^{-2})X^3 - (0.342\times10^{-4})X^4 + (0.636\times10^{-7})X^5$.
$r^2 = 0.64$

$\hat{Y}$ (controls) = $18785.0 - (9.054\times10^{2})X + 16.62\,X^2 - 0.145\,X^3 + (0.613\times10^{-3})X^4 - (0.100\times10^{-5})X^5$.
$r^2 = 0.94$

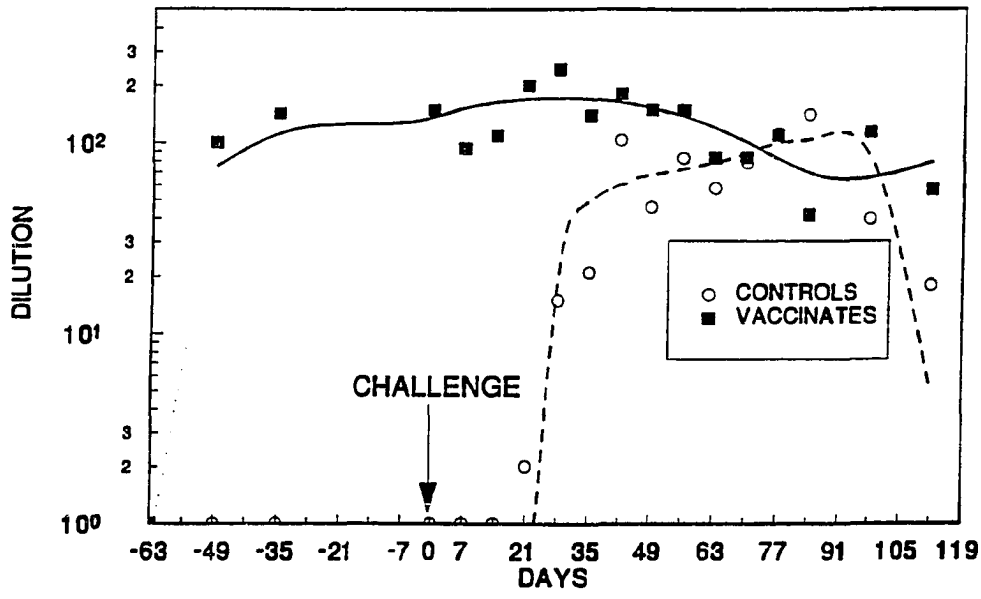

FIGURE 3.2.
Rivanol test results for 7 reindeer vaccinated with *B. suis* 3 vaccine, and 6 non-vaccinated control reindeer. Both groups were challenged with virulent *B. suis* 4 on day 0. Results are presented as geometric mean titers and weighted polynomial regressions.

$\hat{Y}$ (vaccinates) = $-2.655 + 10.25\,X - 0.292\,X^2 + (0.402\times10^{-2})X^3 - (0.256\times10^{-4})X^4 + (0.587\times10^{-7})X^5$.
$r^2 = 0.58$

$\hat{Y}$ (controls) = $75.74 + 3.783\,X - 0.220\,X^2 + (0.321\times10^{-2})X^3 - (0.174\times10^{-4})X^4 + (0.310\times10^{-7})X^5$.
$r^2 = 0.73$

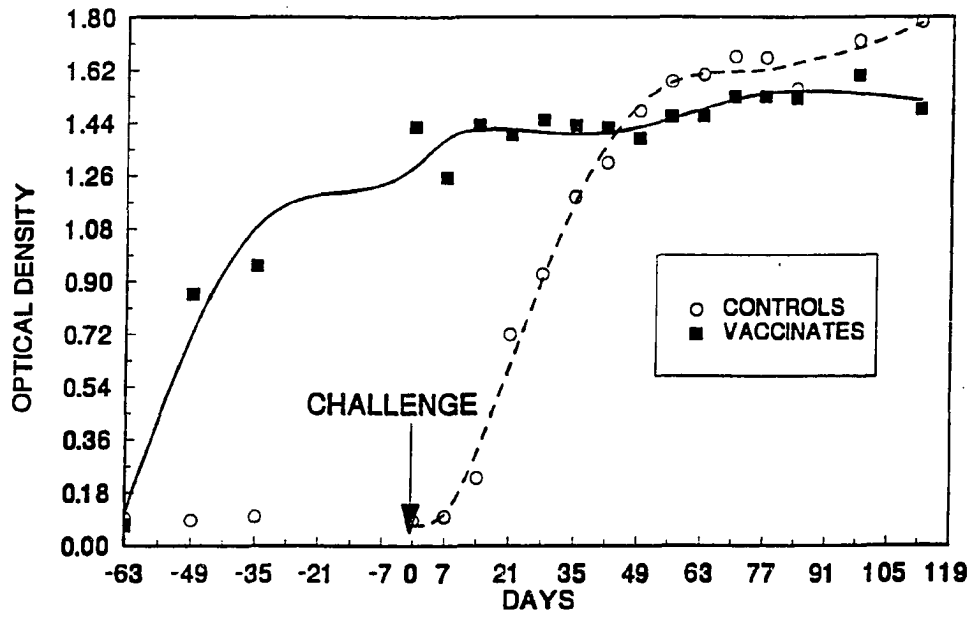

FIGURE 3.3.
ELISA results for 7 reindeer vaccinated with *B. suis* 3 vaccine, and 6 non-vaccinated control reindeer. Both groups were challenged with virulent *B. suis* 4 on day 0. Results are presented as geometric means of spectrophotometric absorbance values with weighted polynomial regressions.

$\hat{Y}$ (vaccinates) = $0.0598 + (0.5987 \times 10^{-1})X - (0.107 \times 10^{-2})X^2 + (0.915 \times 10^{-5})X^3 - (0.358 \times 10^{-7})X^4 + (0.508 \times 10^{-10})X^5$.

$r^2 = 0.97$

$\hat{Y}$ (controls) = $41.64 - 1.98\,X + 0.0355\,X^2 - (0.299 \times 10^{-3})X^3 + (0.121 \times 10^{-5})X^4 - (0.190 \times 10^{-8})X^5$.

$r^2 = 0.99$

Four calves were born to vaccinated dams, 3 of these survived following parturition. A small amount of poor-quality serum was obtained from the calf that died. This serum was negative on the ELISA test. The three remaining calves were serologically positive from the day of birth until the time of necropsy. One of these calves died within 6 days of birth. The other two lived for 32 and 34 days until they were necropsied. These 2 calves were only positive on the ELISA test at the time of necropsy. Milk samples taken from postpartum reindeer were positive on the *Brucella*-ring and whey-plate tests.

Bacteriology

Among the 5 adult vaccinates, 1 aborted a fetus in late March from which *B. suis* 4 was isolated. This reindeer retained her placenta for 36 h. The 4 remaining pregnant vaccinates bore live calves. *B. suis* 4 was not isolated from these calves at necropsy. One of these calves died shortly after parturition. An empty stomach indicated that the calf had never been suckled. Numerous, extensive hemorrhages indicated that the calf had died of trauma inflicted by other reindeer. *B. suis* 4 was isolated from vaginal swabs collected up to 1 month postpartum from the vaccinated reindeer that aborted. No bacteria were isolated from vaginal swabs from other vaccinates.

Positive hemocultures were obtained from 3 of 7 vaccinated reindeer. Bacteremia lasted for 6 weeks in the female that aborted. One positive hemoculture was obtained at 50 days post-challenge from a pregnant female that did not abort and remained culture negative. One other positive hemoculture was obtained from a vaccinated calf that was culture-positive at necropsy.

B. suis 4 was isolated from 3 of 7 vaccinated reindeer that aborted. The uterus, spleen and 11 lymph nodes were culture-positive in the reindeer that aborted. Another vaccinate had a large mammary abscess and 1 mandibular lymph node that were positive

for *B. suis* 4. This reindeer had bore a healthy calf and shown no previous signs of infection with *B. suis* 4. In addition, this animal was thin and lacked sub-cutaneous fat. *B. suis* 4 was isolated from 4 head lymph nodes in 1 of the vaccinated calves. Small subcutaneous granulomas occurred at the vaccination site in 3 of 7 vaccinates.

All 6 mature nonvaccinated reindeer aborted culture-positive fetuses. Abortions occurred from 34 to 74 days after challenge. One of the aborted fetuses was mummified (Appendix 1). Placentas were retained for 2 to 3 days post-abortion in 3 of the control reindeer. *B. suis* 4 was isolated from vaginal swabs of all control reindeer from 4 to 6 weeks post-abortion.

Bacteremia of variable length was seen in all control reindeer. The control reindeer with the shortest bacteremia yielded 2 positive hemocultures 3 weeks apart. The longest bacteremia was seen in the control calf that showed positive hemocultures for 11 continuous weeks. Positive hemocultures were obtained in 49% of 82 blood samples obtained following challenge.

B. suis 4 was isolated from all control reindeer at necropsy. The most heavily infected reindeer (case description in Appendix 1) yielded *B. suis* 4 from 17 of 30 tissues cultured, in addition to 2 peritoneal abscesses. The uterus of this reindeer was filled with thick purulent exudate from which *B. suis* 4 was isolated. *Brucella*-caused carpal arthritis also was observed. Abscesses that were positive for *B. suis* 4 also occurred in one other reindeer. These were associated with the omentum and the wall of the omasum. Both reindeer with internal abscesses were thin and lacked sub-cutaneous fat reserves.

The vaccine was successful in preventing abortion in 4 of 5 pregnant females, and successful in preventing infection in 4 of 7 reindeer (Table 3.1). There was a significant difference ($P < 0.05$) in the infection rate in the vaccinated and control groups using

TABLE 3.1
Culture results indicating infection and/or abortion in *B. suis* 3 vaccinated and control reindeer challenged with virulent *B. suis* 4.

RESULTS	VACCINATES	CONTROLS
Reindeer culture positive for *B. suis* 4 at necropsy/ Total reindeer cultured	3/7 (43%)	6/6 (100%)
Abortions attributable to brucellosis/ Total pregnancies	1/5 (20%)	5/5 (100%)
Calves culture positive for *B. suis* 4 at necropsy/ Total fawns born	0/4 (0%)	

Fisher's exact test (Zar 1984). *B. suis* 4 was isolated from a greater proportion of tissues in control reindeer than in vaccinated reindeer (Table 3.2). Bacterial isolations from hemocultures were more frequent in the control group than in vaccinated group (Table 3.3).

DISCUSSION

The killed *B. suis* 3 vaccine in adjuvant was successful in preventing infection in most vaccinated reindeer (4 of 7). I accept the hypothesis that the *B. suis* 3 vaccine engenders sufficient immunity in reindeer to protect against challenge with virulent *B. suis* 4. The challenge inoculum used in this trial appears to have been particularly virulent. All 6 control reindeer became infected. Numerous tissues were culture-positive in each animal. All 5 pregnant controls aborted fetuses that were positive for *B. suis*. Each control reindeer developed an extended bacteremia. Two of 5 control reindeer developed overt clinical disease, with the formation of abscesses and cachesia. Infection in control reindeer in experiments and with similar challenge doses has not been as severe (Dieterich et al. 1980, 1981, unpubl., Dieterich and Morton 1987).

Results from this vaccine test compare favorably with other challenge trials of brucellosis vaccines in reindeer and other wildlife species. *B. abortus* strain 19 did not provide protection against infection with *B. suis* 4 in reindeer (Dieterich and Morton 1987) or against *B. abortus* infection in bison (*Bison bison*) (Davis et al. 1991). *B. abortus* strain 19 also caused persistent infections and abortions. Three additional vaccines have been tested in reindeer. *B. melitensis* strain H-38 did not provide detectable resistance to *B. suis* 4 infection (Dieterich et al. 1980). *B. abortus* strain 45/20 (Dieterich et al. 1981) protected 5 of 6 vaccinates. A killed *B. suis* 4 vaccine has

TABLE 3.2
Numbers of tissues found to be culture-positive for *B. suis* 4 in control and vaccinated reindeer. Where left and right paired samples were cultured, this is indicated by the number (2).

Tissues culture positive for *B. suis* biovar 4	Controls (n = 6)	Vaccinates (n = 7)
Supramammary lymph nodes (2)	10	2
Mandibular lymph nodes (2)	4	5
Parotid lymph nodes (2)	7	2
Retropharyngeal lymph nodes (2)	3	3
Suprapharyngeal lymph nodes (2)	4	1
Prefemoral lymph nodes (2)	5	0
Uterus	4	1
Abscesses	4	1
Internal iliac lymph nodes	3	1
External iliac lymph nodes	2	1
Prescapular lymph nodes (2)	2	0
Popliteal lymph nodes (2)	0	2
Bronchial lymph nodes	2	0
Udder	2	0
Spleen	1	1
Mediastinal lymph nodes	1	0
Kidneys (2)	1	0
Totals:	55	20

Tissues for which there were no isolations of *B. suis* biovar 4:
Liver
Lung
Heart
Muscle
Mesenteric lymph nodes

TABLE 3.3
Hemocultures positive for *B. suis* 4 in control and vaccinated reindeer in days subsequent to challenge exposure.

Hemocultures positive for *B. suis* 4

Days	Vaccinates	Controls
0	0	0
7	0	0
14	0	4
21	2	5
28	1	6
35	1	2
42	1	4
49	2	6
56	0	3
64	0	4
70	0	2
77	0	2
84	0	1
98	0	0
112	0	0
Totals:	7	39

provided good protection against infection (Dieterich et al. unpubl.). With this vaccine, 75%, 86%, 87% and 92% of reindeer were protected against infection when challenged at 2, 14, 26 and 43 months post-vaccination, respectively.

The *B. suis* biovar 3 vaccine also was successful in preventing abortion in 4 of 5 vaccinated reindeer. One of 2 adult female vaccinates, which became infected following challenge, progressed through a normal pregnancy and bore a healthy calf that was negative for *B. suis*. This calf died 6 days after birth. Trauma was the suspected cause of death. The dam had a *B. suis* 4-positive mammary abscess and mandibular lymph node on necropsy. Lack of nursing in goats enhances localization and replication of *B. abortus* in mammary glands (Meador et al. 1989). Perhaps the lack of nursing from this reindeer contributed towards mammary infection. *B. suis* 4, however, did not become established in the uterus of this reindeer.

Calves have not previously been used in vaccine trials. Russian researchers have considered them to be relatively resistant to brucellosis infection because of a general absence of lesions and antibody titers (Golosov and Zabrodin 1959, Davidov 1961, Orloff 1963). The 3 calves used in this study were 8 months-of-age at the time of vaccination, and 10 months-of-age at the time of challenge; all were reproductively immature. These calves appeared to be as susceptible to brucellosis infection as adults, with 2 of the 3 calves becoming infected. No significant difference ($P = 0.57$) occurred in the infection rate of vaccinated adults (2 of 5) and calves (1 of 2) using Fisher's exact test, but the power to detect such a difference is low. Whether calves of this age are able to mount an immune response following vaccination similar to that of adults is uncertain. Serum antibody levels of the 2 vaccinated calves did not appear to differ from those of adult reindeer. Antibody responses of vaccinated adults and calves would be best compared using data from a large number of animals sampled sequentially over several

years. Such data are available from reindeer herds on the Seward Peninsula that have been serologically monitored following vaccination with *B. suis* 4 (Dieterich, pers. comm.).

All vaccinated reindeer showed a good antibody response to the vaccine. High titers were seen 14 days after vaccination on all serologic tests. Titers in nonvaccinated reindeer that became infected were not as high as those in vaccinated reindeer, particularly on the Riv test. In bovids, this test detects the IgG_1 subisotype (Tizard 1987). Titers in control reindeer were first detected by IgG-specific ELISA and SP tests. The latter test primarily detects IgM in standard bovine serology. Titers as measured by agglutination tests declined over the course of the trial, whereas ELISA results remained stable. Four reindeer maintained high agglutination titers. These included the 2 adult vaccinated reindeer that were infected, and also 2 other unvaccinated adults. The ELISA was most sensitive in detecting humoral responses to infection and vaccination.

The antibody profiles for the 4 calves that were born in this trial were as expected. The calf that died prior to suckling was serologically negative. The 3 calves that survived parturition all showed positive titers. This would be expected with colostral intake. The 2 calves that lived for 1 month prior to necropsy had no detectable antibody as measured by BBA, SP, and Riv tests. ELISA results were positive at the time of necropsy. This is consistent with an expected diminution of passively acquired antibodies over time.

Under the conditions of this challenge experiment, the *Brucella suis* biovar 3 vaccine induced protection against infection and abortion in reindeer. The duration of this immunity beyond 63 days has not yet been determined.

CONCLUSIONS

Brucellosis caused by *B. suis* 4 results in serious losses of productivity in reindeer herds. The disease presents a hazard to persons associated with the handling or butchering of reindeer. Brucellosis in domestic animals is subject to federal regulation. Infected reindeer cannot be transported to other states or to brucellosis-free areas of Alaska. There is a continuing need to control brucellosis in reindeer by: (1) Improving management practices, (2) Optimizing herd immunity through the use of a safe and efficacious vaccine, and (3) Improving diagnosis of the disease.

An effective vaccine, based on killed *B. suis* 4, is currently in use in Alaskan reindeer herds. The main disadvantage to the use of this vaccine is that infected reindeer in vaccinated herds cannot be distinguished from vaccinated, noninfected animals. This has caused difficulties in assessing the prevalences of brucellosis in vaccinated herds, and also in the selection of reindeer for export.

The objective of this research was to provide a vaccine for the control of brucellosis in reindeer that allows serologic discrimination between vaccinated and infected animals. A *B. suis* 3 vaccine was chosen for this purpose because it possessed a different antigenic structure from that of the naturally occurring strain of brucellosis, *B. suis* 4.

An enzyme-linked immunosorbent assay (ELISA) was modified for detection of reindeer antibodies specific for A and M antigens of *B. suis* With this test, 89% of 117 reindeer were correctly classified as either *B. suis* 3-vaccinated or *B. suis* 4-infected.

Discrimination between vaccinated and infected reindeer is sufficient to allow assessment of brucellosis prevalence in vaccinated herds, but not sufficient to identify infected individuals.

The ELISA also will be useful in the diagnosis of reindeer with exposure to *B. suis*. The test was more sensitive than agglutination tests currently employed in the diagnosis of brucellosis in reindeer. The ELISA therefore will provide additional information when used in adjunct to these tests.

The *B. suis* 3 vaccine stimulated significant cell-mediated and humoral responses in *Rangifer*. The vaccine provided significant protection against infection and abortion following challenge with highly virulent *B. suis* 4. This vaccine would be suitable for further large-scale testing in reindeer. This testing would be necessary prior to general application of the *B. suis* 3 vaccine in Alaskan reindeer herds.

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APPENDIX 1.

FETAL MUMMIFICATION IN A REINDEER (*RANGIFER TARANDUS*) INFECTED WITH *BRUCELLA SUIS* BIOVAR 4

ABSTRACT

An adult female reindeer (*Rangifer tarandus*), was challenged with 3.155×10^7 colony forming units of *Brucella suis* biovar 4. One and one-half months after challenge, the reindeer aborted a mummified fetus. Fetal mummification has not been reported previously in association with this disease in reindeer. The reindeer also developed a generalized, terminal *B. suis* 4 infection, with rapid development of carpal arthritis, abdominal abscesses, bronchopneumonia, and hypergammaglobulinemia.

CASE REPORT

Brucellosis due to *Brucella suis* biovar 4 infection has been recognized as an enzootic disease in reindeer and caribou herds in circumpolar regions (Meyer 1966). The main effect of the disease is in reproductive losses from abortions, which occur 1-2 months before normal calving time (Rausch and Huntley 1978, Zabrodin et al. 1980). Abortions are frequently followed by metritis (Zabrodin et al. 1980). Calves of infected females normally are weak and later die (Neiland et al. 1968). Surviving calves can become carriers of brucellosis (Dieterich 1981). Reindeer that have aborted usually carry their calves to term in subsequent years (Nikolaevskii 1961). The disease in males frequently is manifested by orchitis and epididymitis (Golosov and Zabrodin 1959). Inflammation of the accessory sex organs may occur (Dieterich 1981). *B. suis*-caused

arthritis and bursitis are common in both sexes (Orloff 1963). In advanced cases, abscesses occur in mammary glands, reproductive organs, liver, kidney, peritoneum, and subcutaneous tissues (Dieterich 1981). I describe a generalized, terminal *B. suis* 4 infection in a reindeer, with the unusual features of fetal mummification, early abscess development, bronchopneumonia, and development of hypergammaglobulinemia.

A 6-year old female reindeer, with no serologic evidence of exposure to *B. suis* 4 was brought into isolation facilities at the University of Alaska Fairbanks. This animal served as a control in a vaccine efficacy experiment. The reindeer was challenged on 25 January 1989 with 3.155×10^7 colony forming units of *B. suis* 4. The inoculum had previously been passaged through guinea-pigs and lemmings to assure virulence.

Diarrhea was first noted at 22 days post-inoculation, and lasted 1 week. *B. suis* 4 was isolated from blood samples drawn on days 22 and 29. At 40 days post-inoculation, recurrence of diarrhea was seen in conjunction with depression and listlessness. A greenish, mucoid vulval discharge also was noted. A complete blood count (CBC) and fecal analysis revealed a moderate neutrophilia and a heavy strongyle infection. The reindeer was treated with ivermectin.

The reindeer aborted a mummified fetus 44 days post-inoculation. Advanced autolysis precluded identification of most internal and external features of the fetus. Tissue samples identified as heart, lung and liver were cultured on both trypticase soy agar[1] plates and a selective media containing per liter: 25g tryptose broth, 20g of agar[2], 0.15ml of Tergitol 7, 25ml of Tween 40, 1.4 mg of ethyl violet, 1.44g of sodium lauryl sulfate, 1 vial of CNV[3] , and 500mg of cylcoheximide[4]. Heart and lung yielded almost pure cultures of *B. suis* 4. No bacterial growth was associated with the liver tissue. The

1 Trypticase Soy Agar, Baltimore Biological Laboratories, Cockeysville, Md.
2 Bacto Agar, Difco Laboratories, Detroit, Mich.
3 Cholestimethate, nystatin, vancomycin, Difco Laboratories, Detroit Mich.
4 Actidione, Sigma Chemical Co., St. Louis, MO

reindeer continued to shed *Brucella* organisms per vagina until it was euthanized 6 weeks later.

Other causes of abortion were ruled out by serologic testing[5] of blood samples collected prior to challenge, at the time of abortion, and 14 days after abortion. None of these samples contained serologic evidence of exposure to infectious bovine rhinotracheitis, bovine viral diarrhea, or leptospirosis.

The reindeer regained an alert demeanor after the abortion. Nonetheless, it began to lose weight and developed a swollen left carpus 50 days post-inoculation. The carpus continued to enlarge until the 78th day post-inoculation, when the synovial membrane and skin ruptured and drained a thick purulent exudate. Cultures taken from the carpus were positive for *B. suis* 4. At 85 days post-inoculation, a large prepubic swelling in the abdominal body wall was noted, as well as a swelling on the fetlock of the left foreleg. The large prepubic swelling was a *Brucella*-positive abscess that broke open at 90 days post-inoculation, draining copious amounts of purulent material. The reindeer was euthanized at this time for humanitarian reasons because it was cachectic and listless.

On necropsy, generalized pathologic changes related to brucellosis infection were evident. The parietal and visceral peritoneum were milky with fibrin deposition. Generalized adhesions within the peritoneal cavity bound visceral organs together, with numerous attachments to the parietal wall. Four pleural fibrous adhesions were also evident. The uterus was filled with inspissated suppurative exudate. Two 14-cm diameter abdominal abscesses were observed. One was attached to the omentum. The other was associated with the body wall just posterior to the umbilicus and had been draining to the exterior. The kidneys were pale, enlarged and firm.

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Histologic examination of the kidney revealed chronic glomerular nephropathy and interstitial nephritis. There was proliferation of the glomerular mesangial cells, thickened glomerular basement membranes, marked thickening of Bowman's capsule with hypertrophy of the parietal epithelium, and variable glomerular sclerosis. Few leucocytes were in glomerular tufts. Several tubules contained hyaline casts, few leucocytes, and a few sloughed epithelial cells. Primarily in the cortex but also in the medulla, the interstices were expanded by numerous lymphocytes, which multifocally effaced renal parenchyma.

In the lung, there was mild to moderate, chronic-active, multifocal bronchopneumonia. Lymphocytes and neutrophils were predominantly located in peribroncholar interstitium. Small cuffs had formed around bronchi. Additional lesions observed histologically were mild multifocal cholangiohepatitis, hepatic and splenic hemosiderosis, and lymphocytic depletion in the spleen.

Brucella suis 4 was isolated from 19 of 32 tissues cultured. These included the right (R) prefemoral, R prescapular, left (L) mandibular, paired parotid, paired suprapharyngeal, R retropharyngeal, paired supramammary, mediastinal, paired internal iliac, and external iliac lymph nodes and the spleen, right kidney, uterus, and 2 abscesses.

The serologic response of this reindeer to challenge and infection with *B. suis* 4 was delayed compared with other control animals in this and other similar experiments. Standard plate (SP), buffered *Brucella* antigen (BBA) and rivanol (Riv) tests were conducted on a weekly basis using standard procedures (U.S. Department of Agriculture not dated, b, c). Positive test results were obtained on the BBA at 36 days post-inoculation, the SP at 22 days post-inoculation, and the Riv at 71 days post-inoculation. Antibody titers gradually increased over the course of the infection. Five similarly challenged reindeer developed *B. suis*-specific antibody much more rapidly. All

serologic test results for these reindeer were positive at day 22 for the BBA, day 14 for the SP, and day 36 for the Riv.

Serum samples, which were taken from the subject reindeer pre-challenge and 85 days post-challenge, were evaluated by serum protein electrophoresis[6]. Sera from 21 healthy Seward Peninsula reindeer served as controls. These samples were obtained in June. Serum protein electrophoresis has revealed seasonal and age variations in the blood proteins of *Rangifer* (Nieminen et al. 1980, Nieminen and Timisjarvi 1983). Results of these tests (Table 4.1, Figure 4.1) show that the subject reindeer developed a hypergammaglobulinemia, with gamma 1 globulin levels of 3.62 g/dl, and low serum albumin concentrations, with albumin levels of 1.67 g/dl. Albumin/globulin of this reindeer was 0.29 as opposed to the mean of 1.57 for healthy reindeer.

Hyperglobulinemia attributable to increased gamma and beta 2 globulin concentrations as shown in Figure 4.1 is compatible with increased immunoglobulin production. In this case the immunoglobulin increase is likely to be an IgG, IgM response associated with persistent antigenic stimulation from *B. suis* 4. The hypoalbuminemia, which accompanies the hyperglobulinemia, may be due to several causes. A compensatory decrease in concentration of serum albumin occurs with hyperglobulinemia. Also, cachexia associated with chronic inflammation results in decreased albumin production, and albumin may have been lost into the urine through glomerular lesions.

Necropurulent brucellosis lesions have traditionally been regarded as a chronic manifestation of the disease in reindeer, occurring 6 months or more after infection (Nikolaevskii 1961, Zabrodin et al. 1980). The subject reindeer developed carpal

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TABLE 4.1.
Blood protein electrophoretic values of 21 adult female Seward Peninsula reindeer as compared with pre-inoculation and pre-mortem samples taken from case reindeer.

Reindeer	alpha1	alpha2	beta1	beta2	gamma1	gamma2
Seward Peninsula						
Mean		0.22	0.2	0.58	0.55	1.00
Max		0.33	0.29	0.94	0.92	1.27
Min		0.12	0.14	0.16	0.31	0.51
Error		0.04	0.05	0.24	0.25	0.24
Case Reindeer						
pre-inoc	0.37	0.14	0.17	0.60	0.68	1.18
pre-mort	0.38	0.16	0.20	1.36	3.62	

Reindeer	glob[1]	pro[2]	alb[3]	alb/glob
Seward Peninsula				
Mean	2.54	5.60	3.39	1.57
Max	3.75	6.40	3.87	1.86
Min	1.24	4.80	2.81	1.04
Error	0.82	0.54	0.37	0.24
Case Reindeer				
pre-inoc	3.14	6.90	3.75	1.19
pre-mort	5.72	7.40	1.67	0.29

[1] globulin
[2] protein
[3] albumin

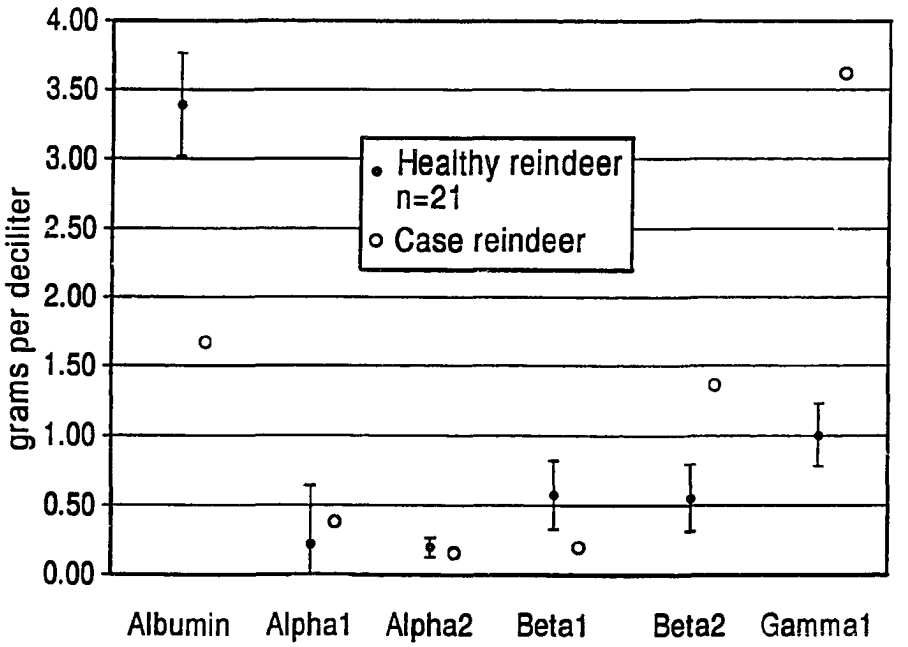

FIGURE 4.1.
Serum protein electrophoretic values for 21 healthy reindeer and subject reindeer expressed as a mean ± SE for each protein fraction.

arthritis within 2 months of infection, and a large, draining abscess within 3 months. Evidence suggests that in a severe infection or under heavy challenge, the formation of brucellosis lesions is expedited. Three reindeer that Davidov (1961) challenged with 10^8,10^9 and 10^9 *B. suis* 4 organisms developed necropurulent epididymitis, mastitis, and bursitis, respectively, approximately 1 month after challenge. Experience with a challenge dose of 10^7 CFU of *B. suis* 4 in other similar experiments suggests that it should not be considered a heavy challenge. This dose does not usually cause a severe infection in reindeer.

Abortion is a common symptom of brucellosis in reindeer. Fetal mummification has not been reported previously. One report (Rausch and Huntley 1978) details the abortion of a mascerated fetus under similar circumstances. Bacterial culture of this fetus was not attempted. Thus the cause of abortion remains unclear. Fetal mummification has been seen in swine and dogs in association with brucellosis infection but has not been reported in the literature (Deyoe, pers. comm.).

Bronchopneumonia is a common manifestation of aborted animals infected with brucellosis. This finding, however, is unusual in infected adult animals.

Brucellosis in reindeer is considered a disease of high morbidity but low mortality (Nikolaevskii 1961, Rausch and Huntley 1978, Zabrodin et al. 1980, Dieterich 1981). I believe that the subject reindeer would have died of brucellosis. Perhaps the case fatality rate in reindeer herds has been underestimated. Certainly, *B. suis*-caused arthritis and bursitis, and also the lack of vigor associated with severe, generalized infection would make reindeer more susceptible to predation. Under herding systems presently used in Alaska, the loss of these animals would go unnoticed.

Also, this case is notable in that this reindeer shed *Brucella* organisms: (1) In vaginal exudate, (2) From an infected joint, and (3) From a draining abscess. This drainage persisted over 2 months. Such a reindeer would present a large infection risk for other reindeer it was in contact with and would be a major source of ground contamination.